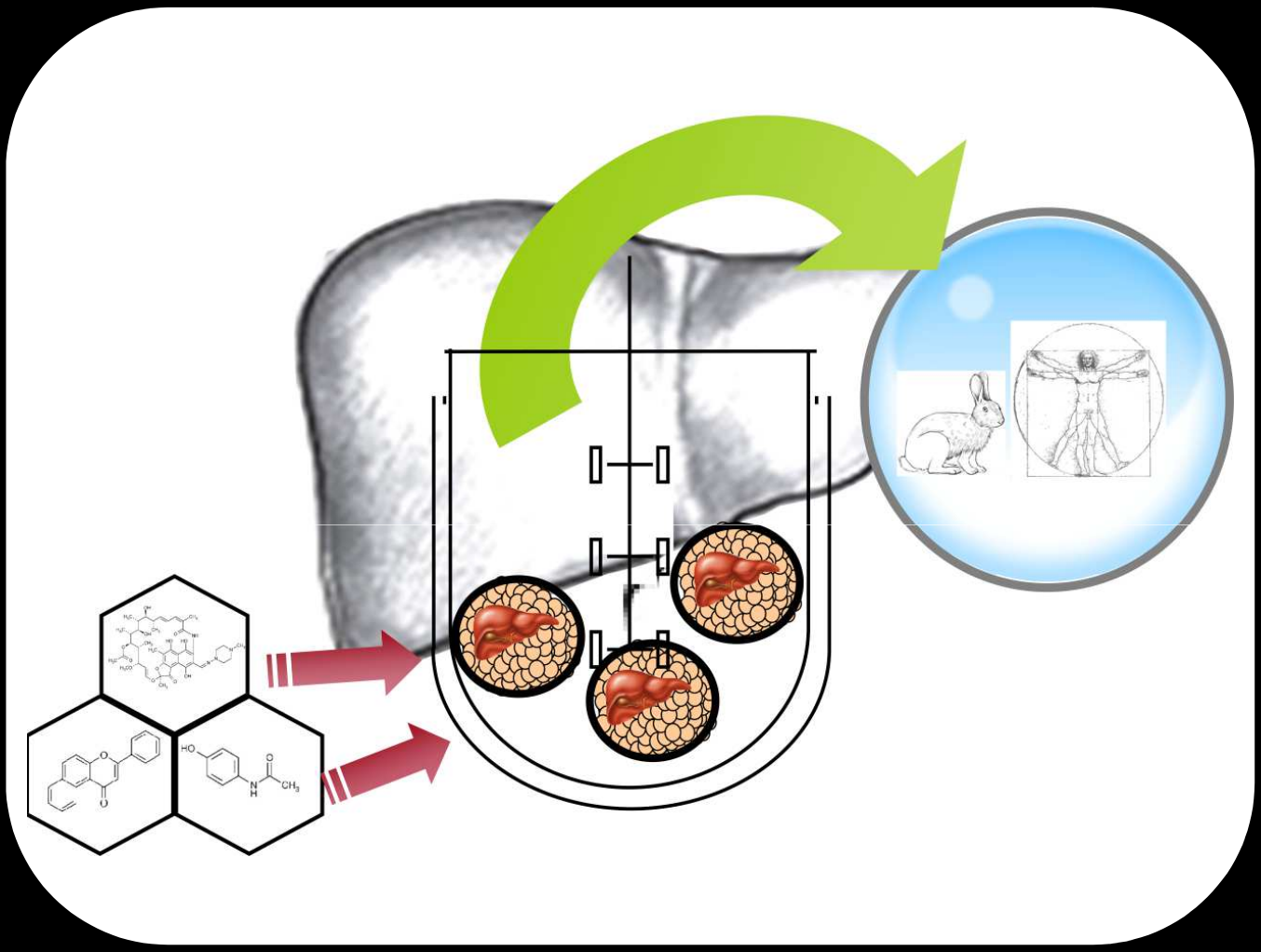
Novel Approaches for Culturing Hepatocytes for Drug Testing Applications

Sofia Margarida Batista Leite



Dissertation presented to obtain the Ph.D degree in Biochemistry, Biotechnology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
March, 2012

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA /UNL

Knowledge Creation

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by Sofia Batista Leite

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Compounds on the left: β-Naphtoflavone; Rifampicin and Acetaminophen

ITQB-UNL/IBET Animal Cell Technology Unit

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Foreword

The present thesis dissertation is the result of four years of research at the Animal Cell technology Unit of ITQB-UNL/IBET, Oeiras, Portugal, under the supervision of Dr. Paula Alves. The work described on Chapter V was performed at the Alternative Methods Unit of Institute for Health and Consumer protection at the EC JRC-Ispra, Italy. All together, it gave me the opportunity to be introduced to the field of Biotechnology and Toxicology to culture hepatocyte cells in stirred tank bioreactors towards the development of alternative methods for animal testing.

This thesis pretends to explore the use of stirred tanks with and without environment control on the improvement and extended maintenance of liver-specific functions on hepatocytes cultured as 3D spheroids. For that rat hepatocytes in mono- and co-culture, human hepatocytes and HepaRG cells were used.

Aos meus pais

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I would like to express my sincere gratitude to all people that direct or indirectly helped during this thesis.

First of all, I would like to thank my supervisor, Dr. Paula Alves, for the inspiration on dedication, to fight for the causes and for showing me the humanized side of Science. For motivating me to embrace the journey of a PhD. For the support, discussion and constant challenges during these 4 years of work. For encouraging me to perform better and being a better student in a whole. For making me see that as good as we can be as individuals we can never be better than a team. For giving me the opportunity to work abroad and became a better researcher and a enriched person. And finally for leading the challenging boat that is the Animal Cell Technology Unit.

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thank to Joana Baptista for all the sharing. To Ligia, Vera and Ana Patricia for all the dinners and funny breaks.

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To my ACT good friends, starting with António, Maria João, Guida, Cristina Peixoto and Sofia A. For the help in the lab, for the comprehension and collaboration, for being my friends.

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Á minha familia, tios e primos, por sermos o que somos, por nos provocarmos sempre a ser melhor e por no fundo nunca haver quimico possivel de diluir a cola que nos une. Em especial aos meus avós Mário, Imperatriz e à memória da minha avó Maria, por todo o orgulho e por me fazer ver que nem sempre quando partimos maçãs saímos a perder.

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ABSTRACT

Drug Development is a long and costly process that starts with thousands of drug candidates and ends with one compound, which often fails in clinical trials. This high percentage of failure of the drug development process results from the lack of highly predictable models in the pre-clinical tests.

Being metabolism a bottleneck in *in vitro* drug testing, special efforts have been made towards the development of metabolic competent liver cell cultures. The major drawback of standard hepatocyte cultivation systems is the spontaneous cell dedifferentiation that results in the decrease of metabolic competency and other liver specific functions and ultimately will compromise the effective biotransformation of drugs. Therefore, novel culture strategies presenting an improved hepatocyte viability and functionality for extended periods are required.

Within this context, the main goal of this PhD project was to develop an efficient strategy for culturing hepatocytes as 3D spheroid structures since this better mimics the *in vivo* tissue. Within these 3D structures, cells can re-establish cell-cell interactions and specific microenvironments resulting in a better retention of important hepatic functions.

The work here depicted has focused on the use of primary cultures of hepatocytes, since they better retain the liver functional profile. Additionally, the developed strategy has been applied to a human hepatocarcinoma cell line with the aim of generating a more physiological model with cells that are easier to obtain then fresh isolated hepatocytes. Within this context, and also to confirm the robustness of the 3D culturing strategy, three relevant cellular models were used namely, human hepatocytes, rat hepatocytes and the HepaRG cell line.

In **Chapter I** the importance of having alternative methods to animal testing, regarding both the ethical problems and the inter-species variances, is explained. Furthermore, the relevance of having metabolic competent methods towards the accomplishment of the 3R's is discussed, highlighted the advantages of having 3D liver cultures for toxicity tests. Special focus is given to the bioreactors used for this purpose, which are a robust and controlled way of cell maintenance.

Although primary cultures of human hepatocytes are the best culture model for liver cells, their use still relies on scarce sources. Thus, the novel strategy developed during the course of this thesis was firstly optimised using rat hepatocytes as described in **Chapters II** and **III**. Moreover, the development of highly predictable cultures of rat hepatocytes is also a way to reduce and refine the animal testing.

In **Chapter II,** the identification of critical parameters used to develop the 3D hepatocyte culture in stirred tanks is described, namely the testing of different stirring impellers, culture media and cell inoculum. The best strategy – culturing rat hepatocytes in 3D spheroids using a paddle impeller, William's E medium with an inoculum of $1{,}2 \times 10^5$ cell/ml – has shown to improve specific hepatocyte functions up to 10x and also extend it to 3 weeks when compared with the 1-2 weeks of the 2D cultures. Also, the cells were further challenged to perform the physiological phenomenon of drug clearance.

The system was then tested for further improvement. Aiming at creating a more physiological milieu, other cell types were introduced into the system, more specifically fibroblasts were co-cultured with rat hepatocytes as described in **Chapter III**. The study started by testing different parameters such as type of fibroblasts, ratio between the concentration of the two cell types and the total cell inoculum. As expected, it was confirmed that over the same period of time, co-cultures had a higher and more stable albumin production as well as improved phase I and II enzymes activities. The best results were obtained for co-cultures of hepatocytes with mouse embryonic fibroblasts in a ratio of 1:2 with a total inoculum of $1{,}2 \times 10^5$ cell/ml. Moreover, CYP induction under different oxygenations (reflecting the heterogeneous hepatic exposure to oxygen *in vivo*) was observed, confirming the biotransformation capacity of the cells.

After the optimisation of the system, **Chapter IV** describes the implementation of a 3D culture of human hepatocytes in a perfusion system using cells from different donors. Besides the expected inter-donor variability, culturing cells under the described conditions has been shown to be highly reproducible regarding albumin and urea secretion. Additionally, hepatocytes have shown CYP inducibility and re-

inducibility after 2 to 4 weeks for CYPs 1A2, 2C9 and 3A4. Moreover, immunostaining of the cell spheroids has shown that they present an *in vivo* like structure with biliary structures and polarised arrangement.

Chapter V describes the generation of a more physiological culture of HepaRG cell lines as an attractive alternative to the use of primary cultures of human hepatocytes. This cell line has shown a biotransformation activity within the range of what is observed for human cells (both *in vitro* and *in vivo*) and for a longer period, up to 7 weeks (a great improvement when compared to the 3-4 weeks longevity observed for the 2D HepaRG cultures). Moreover, the application of the system to screening toxicological studies and to a multidisciplinary integrative model, has been shown. The test of Acetaminophen toxicity in 3D spheroids generated with a stirred tank and using 96-well plates, suggests that the developed culture model is a potential model to be used as a cell-feeder system for high-throughput assays. Furthermore, the integration of these results in a computational model has shown the flexibility of the system to integrate with in silico approaches, contributing to the generation of animal replacement strategies for toxicology applications.

Chapter VI consists of a general discussion integrating all the results described in the previous chapters and the state-of-the-art of hepatic cultures. The main achievements of the work are discussed, namely the generation of a more predictable metabolic model. Final conclusions are then presented as well as discussing the future outlook towards the accomplishment of the 3R's policy.

In conclusion, the 3D spheroid hepatocyte culture systems developed in this work are promising tools that may be used for the establishment of more robust and predictable *in vitro* models for Drug Discovery.

RESUMO

O desenvolvimento de fármacos é um processo longo e dispendioso que começa com milhares de compostos candidatos e termina com uma única, que na maior parte das vezes é reprovada na fase de ensaio clínico. A elevada percentagem de insucessos que ocorrem durante a otimização de fármacos resulta, em grande parte, da inexistência the modelos para teste na fase pré-clinica que sejam mais fiáveis.

Sendo o metabolismo um ponto fulcral dos testes *in vitro*, nos ultimos anos tem vindo a ser feito um grande investimento no desenvolvimento the culturas the células de figado que sejam competentes metabolicamente. O modo tradicional de cultura de hepatócitos leva à sua desdiferenciação expontânea, por decréscimo das funções hepáticas, comprometendo a correta biotransformação dos xenobióticos. Assim sendo, é necessário o desenvolvimento de novas estratégias para a cultura de hepatócitos, de forma a aumentar a sua viabilidade e manter as suas funções específicas durante mais tempo.

Neste contexto, o principal objetivo deste trabalho de Doutoramento foi desenvolver uma estratégia eficaz para cultura de hepatócitos em estructuras 3D que mimetizam melhor o tecido *in vivo*. Neste tipo de estruturas, as células podem restabelecer as interações célula-célula e o microambiente específico do fígado, verificando-se assim uma melhor mimetização do desempenho da função hepática.

O trabalho focou-se essencialmente na ultilização de culturas primárias, tendo em conta que conseguem reter melhor o perfil funcional do fígado. No entanto, esta estratégia foi também utilizada para a cultura de uma linha celular de hepatocarcinoma, de forma a obter um modelo mais fisiológico com células que são mais fáceis de obter que os hepatócitos provenientes diretamente do tecido vivo. Assim, neste contexto e também para confirmar a robustez da estratégia de cultura em 3D, foram usados três modelos celulares relevantes, nomeadamente, hepatócitos Humanos, hepatócitos de Rato e a linha celular HepaRG.

O **Capítulo I** revê a importância da existência de métodos alternativos à experimentação animal, considerando a responsabilidade ética, mas também a

variabilidade inter-espécie entre animais e humanos. É também mostrada a relevância da existência de modelos metabolicamente competentes no sentido de concretizar a politica dos 3R's (Substituir-Reduzir-Refinar, em inglês: *Replace-Reduce-Refine*), mostrando a vantagem de ter culturas 3D de células de fígado, para testes toxicológicos. É ainda dada especial relevância aos biorreatores atualmente usados para este propósito, como modelos de cultura controlados e robustos para manutenção de células.

Apesar das culturas primárias de hepatócitos humanos serem o melhor modelo celular para mimetização *in vitro* do fígado, a sua disponibilidade é bastante limitada. Desta forma, a estratégia de cultura de hepatócitos desenvolvida nesta tese foi primeiramente otimizada usando culturas primárias de hepatócitos de rato descritas nos **Capítulos II** e **III**. Adicionalmente, o desenvolvimento de culturas rubustas de hepatócitos de rato irá também contribuir para a redução e refinamento dos testes em animais.

No **Capítulo II** está descrita a escolha dos parâmetros para o desenvolvimento das culturas 3D em tanques agitados, nomeadamente o efeito de diferentes tipos de pás de agitação, o meio de cultura e o inóculo celular. A estratégia que demonstrou ser a melhor – cultura de hepatócitos em esferoides (3D) em tanque agitado por pás, usando o meio de cultura William's E com um inóculo de células de $1,2x10^5$ cell/ml – demonstrou poder aumentar as funções específicas dos hepatócitos até 10x comparando com as culturas 2D, ao mesmo tempo que foi possível aumentar o tempo de cultura de 2 para 3 semanas. No final foi ainda testada a capacidade das células de desempenhar uma função fisiológica, a depuração hepática.

O **Capítulo III**, o melhoramento do sistema de cultura descrito no capítulo anterior ao introduzir outro tipo de células, neste caso, fibroblastos. O estudo iniciou-se testando diferentes parâmetros nomeadamente o tipo de fibroblastos, a razão entre as duas concentrações celulares e o inóculo celular. Como esperado, para o mesmo período de tempo, os hepatócitos em co-cultura apresentaram uma maior e mais estável produção de albumina, bem como atividade dos enzimas de fase I e II.

A estratégia que apresentou melhores resultados foi a que manteve os hepatócitos em cultura com fibroblastos embrionários de ratinho, numa proporção 1:2 com inóculo total de células de $1,2x10^5$ cell/ml. Adicionalmente, as células responderam à indução das CYPs, dependendo a amplitude de resposta da oxigenação (refletindo a exposição heterogénea a oxigénio característica das células no fígado), confirmando a capacidade de desempenhar a biotransformação.

Após otimização do sistema, o **Capitulo IV** descreve a implementação de culturas 3D de hepatócitos humanos em biorreator de perfusão usando células de diferentes dadores. Apesar da natural variabilidade entre dadores, a manutenção das células em tanques agitados como 3D em condições de cultura controladas demonstrou elevada reprodutibilidade em termos de secreção de albumina e ureia. Em termos de capacidade de biotransformação as células demonstraram capacidade de indução e re-indução após 2 a 4 semanas, das CYPs 1A2, 2C9 e 3A4. Adicionalmente, a marcação por imunofluorescência dos esferoides mostrou uma estrutura semelhante ao fígado *in vivo* com estruturas biliares e organização polarizada.

O **Capítulo V** descreve o desenvolvimento de uma cultura mais fisiológica da linha celular HepaRG, como uma alternativa atrativa ao uso de culturas primárias de hepatócitos. As células demonstraram atividade de biotransformação dentro do mesmo intervalo observado pelos hepatócitos humanos (tanto *in vitro* com *in vivo*) até 7 semanas (em comparação com as 3-4 semanas previamente obtidas com as culturas 2D de HepaRG). Foi também possível demonstrar a aplicação das células em estudos de toxicologia e integração multidisciplinar. O teste de toxicidade de Acetominofen em esferoides gerados em tanques agitados, usando placas de 96-poços, demonstra a compatibilidade do modelo desenvolvido com testes high-throughput. A integração dos resultados gerados em mdelos computacionais demonstrou a flexibilidade do sistema conjuntamente com aplicações *in silico* no sentido de gerar uma subtituição aos testes toxicológicos em animais.

O **Capítulo VI** consiste na integração de todos os resultados dos restantes capítulos desta tese numa discussão geral. São demonstrados os principais

resultados do trabalho desenvolvido originando um modelo metabólico com melhor preditividade; a conclusão geral e as perspetivas futuras.

Em resumo, a cultura de esferoides 3D de hepatócitos desenvolvida neste trabalho é uma ferramenta promissora para a obtenção de modelos *in vitro* mais robustos e preditíveis no desenvolvimento de fármacos.

Thesis publications

1. Miranda JP, **Leite SB**, Muller-Vieira U, Rodrigues A, Carrondo MJT, Alves PM. "Towards an extended hepatocyte *in vitro* culture." Tissue Engineering: Part C, 15(2) (2009): 157-67

2. **Leite SB**, Teixeira AM, Miranda JP, Tostoes RM, Clemente JJ, Sousa MS, Carrondo MJT, Alves PM. "Merging bioreactor technology with 3D hepatocyte-fibroblasts culturing approaches: enhanced *in vitro* models for Toxicological Applications" Toxicology in Vitro 25(4):825-32. (June 2011) [Epub ahead of print]

3. Tostões RM, **Leite SB**, Brito C, Serra M, Jensen J, Bjorquist P, Carrondo MJT, Alves PM. "Human liver cell spheroids in extended perfusion bioreactor culture for repeated dose drug testing." Hepatology (Accepted)

4. **Leite SB**, Wilk-Zasadna I, Zaldivar JM, Airola E, Minnecozzi M, Reis-Fernandes M, Guillouzo C, Chesne C, Alves PM, Coecke S. "3D HepaRG Model for Toxicokinetic and Toxicodynamic Purposes" (submitted)

Abbreviations

Abbreviation	Full form
2D	Two-dimensional / monolayer (cell cultures)
3D	Three -dimensional (cell cultures)
3R's	Replace-Reduce-Refine (animal testing)
ADME	Absorption, Distribution, Metabolism, Excretion
APAP	Acetaminophen
aPKC	Atypical Protein Kinase C
BAL	Bioartificial Liver Device
BNF	β-Naphtoflavone
CDFDA	5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate
CYP	Cytochrome P 450
DAPI	4',6-diamidino-2-phenylindole
Diff	fully Differentiated hepatocytes
DILI	Drug Induced Liver Injury
DMSO	Dimethylsulfoxide
DMEM	Dulbeco's Minimum Essential Medium Eagle's
DO	Dissolved Oxygen
ECM	Extracellular Matrix
ECOD	Ethoxycoumarin O-deethylation
EGF	Epidermal Grow Factor
EGTA	Ethylene Glicol Tetracetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicines Agency
FBS	Foetal Bovine Serum
FDA	(US) Food and Drug Administration
GSTA-1	Glutathione S-Transferase Alpha 1
HCV	Hepatitis C Virus

Abbreviation	Full form
Hep	Hepatocytes
Hff	Human foreskin fibroblast
HGF	Hepatocyte Growth Factor
HNF4α;	Hepatocyte Nuclear Factor alfa;
Lac/Glc	Lactate formation to Glucose consumption Ratio
LDH	Lactate Dehydrogenase
4-MU	4-Methylumbeliferone
MEF	Mouse Embryonic Fibroblasts
MEM	Minimum Essential Medium Eagle's
MCmB	Multicompartmental bioreactor
PB	Phenobarbital
PBS	Phosphate Buffered Saline
PBTK	Physiologically Based Toxicokinetic models
PFA	Paraformaldehyde
Pharma	Pharmaceutical Industry
pO_2	Partial Pressure of Oxygen
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RIF	Rifampicin
RWV	Rotating Wall Vessel
SEM	Standard Error of the Mean
SD	Standard Deviation
TTC	Threshold of Toxicological Concern
TK	Toxicokinetics
UDP	Uridine Diphosphate
U-Diff	Under-differentiation hepatocytes
UGT	UDP-glucuronosyl transferase
UGT2B7	UDP-Glucuronosyltransferase-2B7

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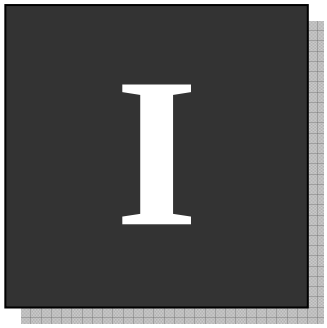


INTRODUCTION

Sofia B. Leite has writen the whole chapter, based on the refered papers.

CONTENTS

1. ALTERNATIVE METHODS TO ANIMAL EXPERIMENTATION: assessment of ADME towards the 3R'S policy

The development of a medical drug takes several years (5 to 10), involving thousands of compounds and costing millions of dollars (**Fig. 1.1**) until it is on the market to be used by patients (innovation.org 2007). The recent advances on genomics, proteomics and computational sciences have given scientists a better understanding of the human body at the molecular level, enforcing the first steps of the drug discovery process on the producing of more effective compounds. However, there is still a high rate of compound failure on the clinical trial and approval phases (Service 2004), either for inducing human toxicity or for not producing the desire/expected effect.

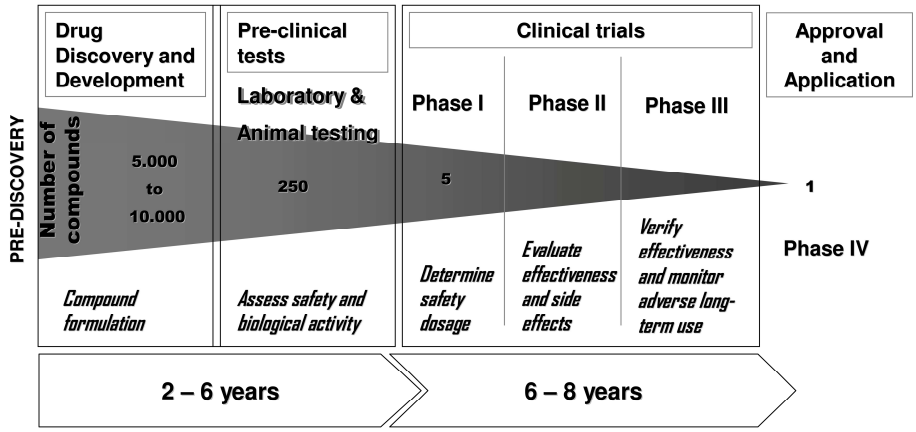


Figure 1.1: Process of Drug Development. (adapted from (innovation.org 2007; Mattisson and Matison Faye 2008))

Besides medical drugs, with the continuous development of the industrialized world, the human body is increasingly exposed to different compounds present in chemicals, pesticides, food or cosmetics. These compounds can enter the body

through different routes reaching distinct targets, and in spite of acute effects being quickly detected, for many of the compounds, chronic toxicity remains unknown.

In this context, the development of methods and strategies that will allow a better prediction of the impact of the new formulations on human health is of capital importance.

Animal models have been used for a long time in order to access the effect of compounds as an integrative ADME (Administration, Distribution, Metabolization and Excretion) model. However, despite the advantages of being an *in vivo* system there are several reasons that have convinced the scientific community of the importance of developing alternative methods. Besides the ethical implications of sacrificing animals for research purposes and the economical impact involved, animal models have been shown to produce false positives regarding the prediction of human toxicity specially at the metabolic level (Turpeinen *et al.* 2009); this is the case with the catastrophic use of Thalidomide and Valpromide in humans.

In 1959, William Russel and Rex Burch introduced the 3R's concept in *The Principles of Humane Experimental Technique* (Russel and Burch 1959), that stands for Refinement, Reduction and Replacement of animal testing. The 3R's concepts were defined as: Refinement – any method that reduces or eliminates pain and distress in animals during experiments; Reduction – methods that seek to use fewer animals in an experimental protocol to obtain the same or similar information of scientific value, or use the same number of animals to obtain more scientifically valuable information; and Replacement – the use of techniques that do not require living animals.

In 1986 a European Directive was established on the protection of laboratorial animals for experimental purposes developing and validation of alternative techniques to animal testing, that latter lead to the creation of ECVAM (European Centre for the Validation of Alternative Methods). Within this scenario, several industries adopted strategies towards the development of alternative methods. REACH (European Regulation on Chemicals) encourages the use of alternative methods and avoidance of tests' duplication; and for Cosmetics legislation a ban has

been gradually introduced on the marketing of cosmetics containing ingredients tested in animals.

However, this year a report prepared for DG SANCO by experts in the field concluded that full animal replacement is still not possible (Adler *et al.* 2011). Nevertheless, contrarily to what is many times assumed, the 3R's policy is not a replacement based strategy. The improvement of alternative methods consists on the development of strategies that can better mimic the *in vivo* system by using less animal sources. The establishment of this policy is based on a set of concepts that have to be considered in order to achieve methods that better predict human toxicity.

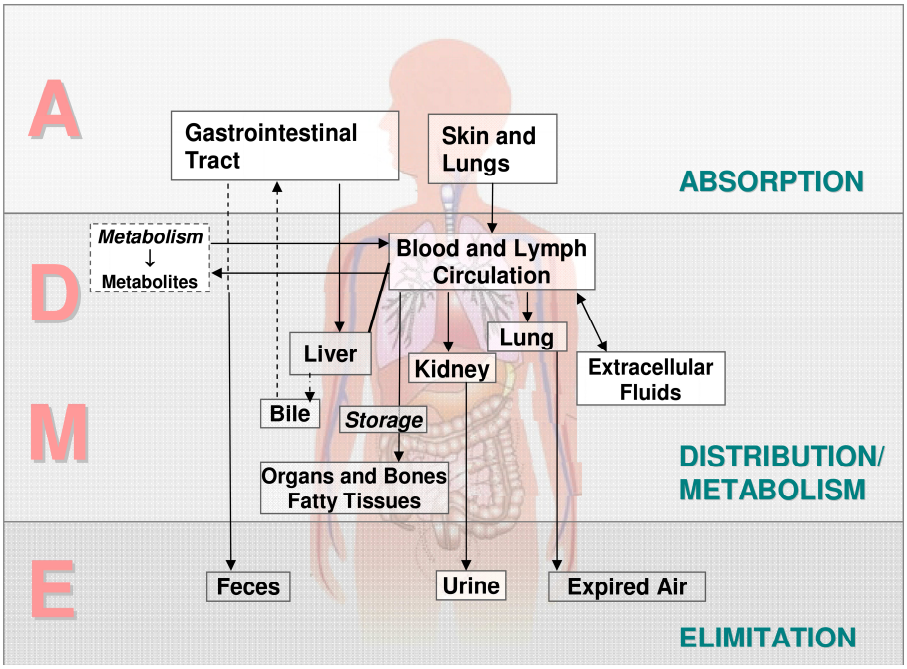


Figure. 1.2: ADME resume of a drug intake, possible paths within the body. ADME (Adapted from Topics on Biotechnology (http://www.biology.iupui.edu/biocourses/Biol540/4pipeline09Full.html) and http://www.humanillnesses.com)

ADME (**Fig. 1.2**) is a concept that more and more has becoming a fulcral point in Drug Development. Many compounds tested in high-throughput screens, often present undesirable features in the clinical phase, namely increased hydrophobicity and decrease solubility (Pelkonen *et al.* 2011). This has made the scientists realize

that ADME testing has to be moved earlier in the drug discover pipeline (Pelkonen *et al.* 2011).

Toxicokinetics (TK) of a compound is the characterization of its ADME, absorption, distribution, metabolism and excretion in the body. Towards the implementation of the 3R's policy, TK has become an essential information that is important to determine parameters such as the bioavailability of metabolites towards the targets; the translation of *in vitro* nominal concentration to the actual *in vivo* levels and to understand if the cell or tissue under human exposure conditions is exposed to the external xenobiotic and/or its metabolite.

With the aim of following the European directives, more reliable methods have been developed and there is already a remarkable set of alternative methods on ADME (**Table 1.1**) some of them have already been validated.

Table 1.1: Main Alternative Methods Available, adapted from (Coecke 2011))

	Test name		Advantages	Limitations
ABSORPTION	*QSAR*	*In silico*	▪ Applicable for dermal and oral exposure. ▪ Able to predict overall absorption of a chemical based on available physicochemical parameters. ▪ OECD principles for the validation of QSARs for regulatory purposes.	▪ Not available for lung absorption. ▪ Active metabolism and transport not yet included
	Artificial intestinal membranes	*In vitro*	▪ Used for very early screening.	▪ Information only on passive diffusion. ▪ Underestimation of the permeability of highly lipophilic drugs.
	Skin Preparations	*In vitro & Ex vivo*	▪ Mainly composed of human source or slaughter sources of animals resembling human (e.g.: pig).	▪ In Skin preparations, the value of absorption under finite dose is specific for the specific exposure time, concentration and skin load making it difficult as input parameter for PBPK-models.
	Cell Cultures	*In vitro*	▪ Standard procedures that can be incorporated in a medium-throughput test strategy. ▪ Allow the study of both absorption and metabolism.	▪ Lack of specificity in vivo metabolic and active transport systems.
	Ussing chamber & Everted sac	*Ex vivo*	▪ Possible to use human sources. ▪ Fast and reproducible study of intestinal absorption of molecules across the inserted tissue. ▪ Allows the study of both absorption and metabolism.	▪ The bioavailability depends on intestinal section. ▪ Limitation on the availability of the test material.
DISTRIBUTION	*QSAR / Computarized models*	*In silico*	▪ Methods still under development, but improving	▪ Predictions not reproducible among different studies. ▪ Poor results obtained for charged molecules under physiological conditions and with charged phospholipids
	Ex-vivo methods (Equilibrium dialysis; Ultrafiltration Ultracentrifugation; Vial-equilibration)	*Ex vivo*	▪ Automation possibility for high-throughput. ▪ Easy to perform; good precision and reproducibility. ▪ Results very close to the ones obtain *in vivo*.	▪ Routine for plasma protein binding, applications for other tissues/organs under development

DISTRIBUTION cont.	*2D and 3D Mixed Cultures*	*In vitro*	▪ Importance of barrier integrity and correlation with in vivo permeability (Pe) and transendothelial electrical resistance recognized	▪ In vitro systems still not adequately characterized for reliable predictions
	Human perfused placenta cotyledon	*Ex vivo*	▪ Minimum number of ethical problems since placentas are discarded after birth. ▪ Up to 48 hr perfusions possible.	▪ The term *placenta* may not reflect the placenta during the first months of foetus development.
METABOLIZATION	*Expert systems QSAR Pharmacophore or molecular protein modelling*	*In silico*	▪ Useful for indicating potential routes and metabolites (but usually overprediction!) ▪ Predictive capability heavily dependent on selected parameters and model compounds	▪ Developed mostly for pharmaceuticals (drug development tools) ▪ Quantitative predictions not still reliable enough
	2D and 3D Cell Cultures	*In vitro*	▪ Studies on metabolic stability, metabolic clearance, metabolite formation; metabolic activation; induction of metabolism and inhibitory interactions with probe substrates. ▪ High-troughput screening and cocktail methods established. ▪ With suitable analytical techniques, covers also elucidation of primary metabolite profile.	▪ Not fully representative of the in vivo specific activities. ▪ Correlations between activation and toxic outcomes not well established. ▪ Limitation on the cells availability. ▪ Missing methods that include the access or real bioavailability/nominal concentration.
EXCRETION	*Computerized models*	*In silico*	▪ Only 4 physico-chemical parameters (charge, molecular weight, lipophilicity, and protein unbound fraction in plasma) are required to predict major excretion pathways.	▪ Still under development. ▪ Not Standardized. ▪ No formal validation studies are known.
	Collagen-sandwich cultures of hepatocytes	*In vitro*	▪ Useful *in vitro* method to differentiate between sinusoidal and canalicular disposition of conjugates.	▪ Not Standardized. ▪ No formal validation studies are known.

The accomplishment of an approved certified alternative method is a process that includes 7 steps: 1) Research; 2) Development; 3) Prevalidation; 4) Validation; 5) Independent Review; 6) Regulatory acceptance and 7) Implementation.

Method validation is part of toxicity testing (Pelkonen 2010). Even being a tedious and time consuming process (5-10 years), the validation of an alternative method, involving several laboratories and a reliable scientific board is a way to ensure the reliability, transferability and robustness of the test method (Pelkonen 2010). This way, the financial and human resources are used more efficiently, having a greater likelihood to meet the expectations of those in the scientific, regulatory and animal welfare communities on alternative methods benefiting and the human society in general.

One of the major drawbacks to achieve viable alternative approaches is the lack of efficient conversion approaches of *in vitro* data, generated at tissue/cell or sub-cellular level, into dose-response information on human body. Within the TK approach, towards the full replacement models, there are several concepts such as Threshold of Toxicological Concern (TTC), biokinetics, IVIVE and integrative approaches that should be taken into consideration (Adler *et al.* 2011).

All together, the alternative methods need then to fulfill the needs and questions of human toxicology, using relevant and competent models and addressing fundamental issues.

When testing the toxicity level of a compound, upfront, is important to determine its TTC value of human exposure: below the TTC there is a very low probability of an appreciable risk to human health (Adler *et al.* 2011). This is a concept that is already being used by the food authorities and is the base of further research projects (Adler *et al.* 2011). If human exposure is above the external TTC it becomes important to access the biokinetics of the compound – the process that the compound will suffer until it reaches its internal concentration and the concentration at which it will reach the target organ(s).

In an *in vitro* approach, besides using the relevant doses, is also important to have bioactivated substances, meaning that the tested substance should be the

substance that *in vivo* would indeed reach the target organ after passing the metabolic organs. It is then important to study the mode of toxic action, and the resulting effects on the physiological processes in an organism. In cell culture it is important to establish strategies that ensure to have enough bioactivated substances *in vitro* such as the use of cell co-culturing, having in culture both the activating cells and the target cells.

First, it is important not only to have competent cell cultures, but ensure also that the cells have a behavior which is as close as possible to the organ itself. To achieve this, several strategies have been developed that will be further explored in the sub-Chapters of Chapter I, but a better predictability will be reached if the cell culture strategy is combined with other approaches and tools. The more extensively characterized a compound is in terms of metabolism, disposition, bioactivation, cell membrane transposition, interaction with molecular processes and cell structures and functions, the higher chances of success in the late stages of the drug development.

The use of *in silico* strategies on kinetic data has been a development strategy used to bridge the *in vitro* and *in vivo*. Today's softwares, can already simulate chemical properties and interactions. When these are used together with other computational models, *in silico* strategies have shown to be the most cost-effective approach (Ekins *et al.* 2010) and to be able to translate the experimental data using the so called Physiologically based Toxicokinetic models (PBTK). To have an efficient *in vivo in vitro* extrapolation (IVIVE) using *in silico* models it is important to have massive generation of data. Thus, it is important to use down-scale and automated models that can screen large amounts of compounds. Metabolic data contributes to explain possible toxicities and modes of action and their relation to dose level and route of exposure are described by Toxicokinetics. Moreover, REACH legislation also requires the testing of thousands of chemicals within a short period of time (few years) (Pelkonen 2010) thus it is of significant importance that the *in vitro* models include or are compatible with high-throughput and high-content approaches

through which a high number of compounds and/or conditions can be screened simultaneously.

While full replacement of animal testing is still not yet accomplished, partial replacement and reduction are forthcoming. For this purpose it is important to develop multidisciplinary strategies that include *in vitro, in silico* and "omics" approaches. A close collaboration between academia, regulatory authorities and industry is of primordial importance. The creation of consortiums, such as DNT (Developmental Neurotoxicity), to discuss and set the necessary path to follow are of utmost importance.

Unlike other toxicokinetic steps, there is still a lack of validated *in vitro* models for metabolization prediction (Adler *et al.* 2011) which is a result of the complexity of maintaining viable and functionally active Liver *in vitro* models.

2. METABOLISM AND BIOTRANSFORMATION

To have a competent *in vitro* toolbox it is important to identify important metabolic pathways of the compound and to provide knowledge on: the metabolic stability of the chemical; the type and structure of metabolites involved. It is also important to determine any metabolism-dependent toxic effects, test the compound's capacity to induce or to inhibit the cell enzymes, check for drug-drug interactions and evaluate its clearance (Coecke *et al.* 2006; Pelkonen *et al.* 2008).

Since metabolism is the bottleneck of *in vitro* toxicology (Coecke *et al.* 2006), it is important to have competent metabolic methods that can accurately determine all the parameters involved on the transformation of a compound before it reaches its target. From the pharmaco-/toxicokinetic point of view, liver is the most important organ in the body. Hepatocytes, present in the liver, have an enzymatic machinery that allows the metabolization of many different foreign chemicals (Coecke *et al.* 2006).

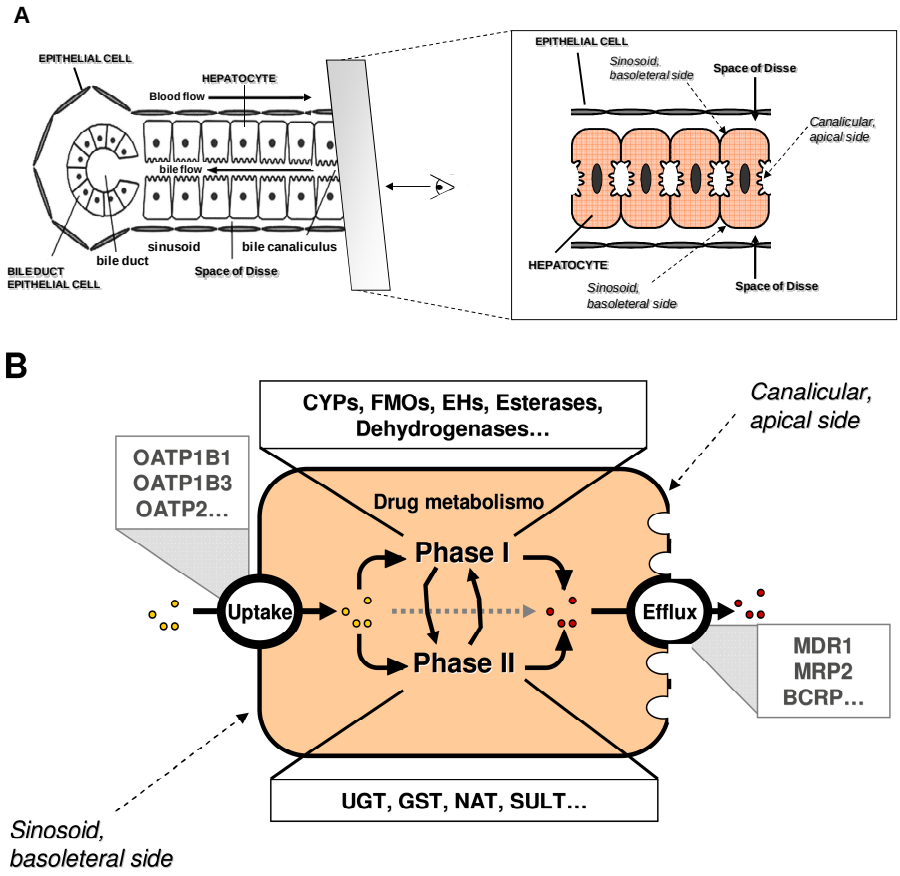


Figure. 1.3. Hepatocyte organization within the liver and specific architecture *in vivo* (Adapted from (Pelkonen *et al.* 2008) and (Dunn *et al.* 1989)). Hepatocytes at the level of functionally important molecules, at the level of tissue architecture, and at the level of kinetic modeling.

Generally, the properties of hepatic cells result from the presence of a comprehensive set of enzymes that guide compounds through a one or two phase process called biotransformation as well as the presence of multiple transporter proteins, some of them with polarized distribution, that perform both influx and efflux functions (**Fig. 1.3 A**) (Pelkonen *et al.* 2008). The polarized structure is a result of the different hepatic surroundings in the liver (**Fig. 1.3 B**), the blood flow is on one side of the cell (sinusoid) and while the bile flow is on the other side (bile *canaliculi*).

2.1 Phase I of Biotransformation

During Phase I of biotransformation the compound (xenobiotic) undergoes oxidative and reduction reactions, carried by monooxygenases becoming more polar. The enzymes involved on phase I are mainly clustered in two groups such as Cytochrome P450 (CYP450), the largest group of membrane associated heme proteins, and flavine monooxygenases. The human CYPs involved in the majority of marketed drugs are CYP3A4, CYP2D6, CYP2C9, and CYP1A2 (Zanger *et al.* 2008).

One of the major drawbacks of the development of liver-predictive models is the high inter-individual metabolic variability. The main cause of these differences is the variation of phenotype and genotype among the enzymes involved in biotransformation, specially the Phase I CYP450 cluster. This is mainly a result of environmental and/or hereditary gene polymorfisms specially on CYPs 2C9, 2C19, and 2D6 (Hewitt *et al.* 2007). On the other hand, although gene expression of CYP 1A1, 2E1 and 3A4 is well conserved, variations on their activity levels can still be observed (Hewitt *et al.* 2007). In this case, the inter-individual differences are the result not only of genetic predisposition but also of many other factors such as hormonal expression, age, sex, diet, smoking, drug consumption, exposure to environmental chemicals and disease state. Among all these factors the interference of other drugs by drug-drug interaction, induction or inhibition is the major and more frequent effect (Hewitt *et al.* 2007). Moreover, the induction of enzymatic capacity has been used as a quality control property, as a way to access the metabolic competence of hepatocytes *in vitro* and thus it has been used as a control factor in validation studies (Richert *et al.* 2010).

The process of enzymatic induction (CYP induction) is the increase of a specific CYP activity upon the addition of specific compound. The classical definition of induction is the *de novo* synthesis of an enzyme (protein) reflecting an increased transcription of the correspondent gene following a specific stimulus. In more detail, once it reaches the cell, the inducer compound will act on the cytosolic receptor ligand which will travel into the nucleus after releasing repressor proteins binding to

the upstream regulatory elements on CYPs (O'Brien 2004). Among the transcription factors, the nuclear receptors such as androstane receptor (CAR), the pregnane X receptor (PXR) as well as the aryl hydrocarbon receptor (AhR) can be distinguished as the main regulators of CYPs activity, acting as sensors for lipophilic xenobiotics, including drugs (Handschin and Meyer 2003; O'Brien *et al.* 2004).

As explained before, induction is a complex process involving several cellular machineries, thus the addition of specific known inducer compounds is a good tactic to check the quality of the metabolic competence of a system for more than one day (Coecke *et al.* 2006). Furthermore, induction has very specific characteristics such as being tissue-specific, rapid, dose-dependent, and reversible upon removal of the inducer (Handschin and Meyer 2003).

However the capacity to respond to one or other compound as well as the fold increase of the enzyme activity is not only species dependent but also donor-dependent (Richert 2009).

Table 1.2 resumes the main xenobiotic CYP metabolizers in human and rodent species, as well as the specific inducers and receptors involved in the induction. It can be observed that the same compound does not always induce the same CYP in one or another species. This differences between species is due sequence differences in the ligand domain of the nuclear receptor genes and CYP response elements (Lin 2006).

Table 1.2: Human and rodent main CYP P450 isoforms with the respective inducers and receptors associated. Adapted from (Handschin and Meyer 2003) and (Ogu and Maxa 2000; Pascussi *et al.* 2000; Borlak *et al.* 2002; Brandon *et al.* 2003; Meredith *et al.* 2003; Tamrazi *et al.* 2003; O'Brien *et al.* 2004; Marek *et al.* 2005; Dail *et al.* 2007; Chanda *et al.* 2009; Richert *et al.* 2009)

P450 isozyme		Inducer compound	Receptor associated
Human	CYP1A1/2	β-Naphtoflavone Omeprozole 3-Methylcholantrine Aroclor 1254	AhR
	CYP2A6	Rifampicin	PXR
		Phenobarbital	CAR
		Pyrazole	ER *(estrogen receptor)*
	CYP 2B6	Rifampicin	PXR
		Phenobarbital	CAR
		β-Naphtoflavone	AhR
	CYP 2C9/19	Rifampicin Dexamethasone	PXR
		Phenobarbital	CAR
	CYP 2E1	Rifampicin Isoniazid	PXR
		Phenobarbital	CAR
	CYP 3A4	Rifampicin Dexamethasone Dieldrin	PXR
		Phenobarbital	CAR
		Omeprazole	AhR
	CYP 4A	Fenofibrate Methylclofenapate	PPARα *(Peroxisome proliferator-activated receptor alpha)*
Rodent	CYP 1A1	β-Naphtoflavone Thiabendazole	CAR
		Aroclor 1254	AhR
		Isoniazid	PXR
	CYP 2A5	Pyrazole	ER *(estrogen receptor)*
	CYP 2B1/2	Phenobarbital Dieldrin	CAR
		Aroclor 1254	AhR
	CYP 2C	Dexamethasone	PXR
	CYP3A1/2	Dexamethasone Pregnenalone-16-Carbonitrile	PXR
		Aroclor 1254	AhR
	CYP4A1	Methylclofenapate	PPARα *(Peroxisome proliferator-activated receptor alpha)*

2.2 Phase II of Biotransformation

After the oxidative pathway of phase I, the xenobiotic can then be excreted or, if it is still not polar enough, it will undergo the conjugation process – Phase II of biotransformation. Conjugation path consists of reactions of glucuronidation, sulfation, methylation, acetilation and mercapture formation, at the end of which more soluble metabolites are obtained; these are ready to be eliminated (Gomez-Lechon *et al.* 2006).

Phase-II metabolization of a compound is dictated by its chemical properties. Within phase II reactions, glucoronidation represents more than 35% of the conjugation reactions in human drug metabolism by the UDP-glucuronsyltransferase (UGT) family of enzymes (Trubetskoy *et al.* 2007). UGT enzymes catalyze the conversion of substrates (exogenous or endogenous compounds) into more polar glucuronides by covalent linking (conjugation) to UDP-glucuronic acid (UDPGA) (Chen *et al.* 2003; Donato *et al.* 2010).

Recent studies have shown that these enzymes can also be induced by some of the prototypical CYP inducers following the same path of ligand-activated transcription factors (Donato *et al.* 2010).

The physiological function of biotransformation is to detoxify the body from foreign compounds. However, the metabolites of biotransformation are often highly reactive and toxic causing hepatotoxicity, being sometimes more toxic than the parent compound (Gomez-Lechon *et al.* 2006). For this reason, in a toxicological test it is important to access toxicity of the compound as well as its metabolite.

The biotransformation capacity is dependent not only on the activity of the enrolled enzymes but also on the activity of the membrane transporters with polarized distribution, for instance, apically transport bile acids on the basal site conduct trafficking of metabolites from the bloodstream. The main superfamily of transporters is the Multidrug resistance-associated superfamily-2 (MRP2) that is a drug efflux pump located on the biliary pole of hepatocytes (**Fig. 1.3 B**) (Courtois *et*

al. 2002). MRP2 belongs to the ATP-binding cassette (ABC) protein superfamily together with other relevant transporters such as MRP1, MRP3 and P-glycoprotein (PGP), the latest also localized on the hepatic biliary side (Borst *et al.* 2000). As it happens with the CYP enzymes, the pump transporter enzymes also respond with increased activity upon the addition of inducer compounds such as dexamethasone and Phenobarbital (Courtois *et al.* 2002).

The role of biotransformation within the organism and the variability associated to it show how important it is to have, in the drug development process, earlier models that behave as close as possible to what is observed *in vivo*, with all the hepatocyte specific functions operating in a reproducible and stable mode. Having good reproducible liver models is an important step forward towards the accomplishment of the 3 R's policy.

3. HEPATOCYTE CULTURES

Besides biotransformation, hepatocytes are responsible for many other body functions that contribute to the regulation of the body homeostasis and to the detoxification of incoming compounds. Under external stimuli (e.g. hormones, nutrients, ions) the hepatocytes secrete and degrade a large number of molecules. The main functions of hepatocytes (besides biotransformation), schematically represented on **Figure 1.4** (De Maria *et al.* 2008) are:

- Regulation of the metabolism of carbohydrates, proteins and lipids and their levels in the blood;
- Synthesis of important serum proteins such as albumin, protrombin, fibrinogen, etc., (with the exception of immunoglobulins);
- Storage of glycogen, triglycerides, iron, copper and liposoluble vitamins;
- Catabolism of endogenous substances, such as hormones and serum proteins, to maintain a balanced body concentration;
- Excretion of the liver metabolites such as conjugated bilirubin, biliar acids, phospholipids, cholesterol and electrolytes to the bile *canaliculum*.

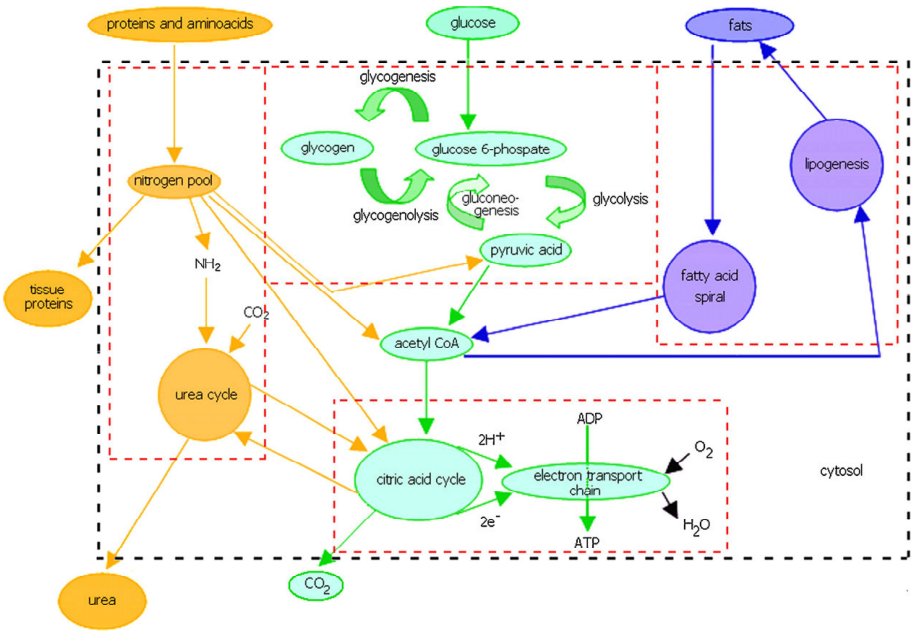


Figure. 1.4. Metabolic hepatic functions (Adapted from (De Maria *et al.* 2008))

When in culture, the maintenance of these functions is attempted by culturing, under chemical defined conditions, appropriate culture medium and supplements and a robust cell culture apparatus. However, it is first of all important to choose the most adequate cell model. Extracellular signals play an important role in the preservation of differentiated liver-specific functions. Within this context, for years researchers have been trying to decrease the spontaneous de-differentiation of *in vitro* hepatocytes by mimicking the extracellular signals using different strategies such as modification of the culture conditions (such as the use of hormonally defined and highly enriched media), addition of soluble factors (such as hormones, growth factors, cytokines and amino acids) (Feldhoff *et al.* 1977; Coecke *et al.* 1999; Schmelzer *et al.* 2009; Takeba *et al.* 2011), increase of cell-cell contacts (Pampaloni *et al.* 2007), addition of matrixes (such as collagen, matrigel, etc.), (Dunn *et al.* 1989) and better control of oxygen levels (Stevens 1965).

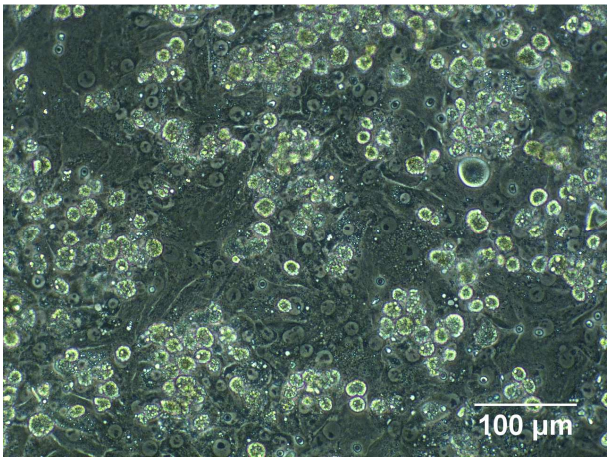
Specific medium components such as Serum, Dexamethasone, Insulin, Glucagone and Growth Hormone have shown to have high impact on the physiological maintenance of hepatocytes *in vitro* (Coecke *et al.* 1999; Schmelzer *et al.* 2009), however some of these factors can also decrease and/or suppress important hepatic machineries such as CYP activity, transporters or protein synthesis (Coecke *et al.* 1999). The solution is to try to balance the importance of the addition of each component according to the aim of the study.

When reproducing the *in vivo* environment in cultured hepatocytes, cell-extracellular membrane (-ECM) interactions, soluble growth factors and cytokines, physical factors (e.g. stress and strain) and cell-cell communications (Lang *et al.* 2011) are important elements that need to be controlled.

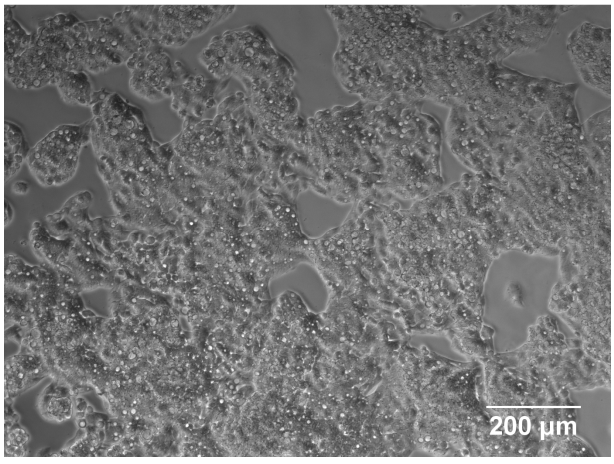
Oxygen is another sensitive issue in hepatocyte cultures. In the liver, the oxygen content on the blood flow from the periportal to the perivenous hepatocytes decreases from 8-9% to 3-5% which will affect the enzymatic profiles of the hepatocytes in each region (Stevens 1965; Jungermann and Kietzmann 1997; Kietzmann and Jungermann 1997; Kidambi *et al.* 2009; Yan *et al.* 2010) leading to the so called liver zonated metabolism (Kietzmann and Jungermann 1997; Allen *et al.* 2005). Several studies state that regular cellular cultures are over-oxygenated (Kidambi *et al.* 2009; Yan *et al.* 2010) since cells are exposed to the 21% of oxygen present in the air while in the liver the oxygen levels are much smaller (Kietzmann and Jungermann 1997), and this could cause oxidative stress. On the other hand, some works defend that cells are exposed to an unlimited oxygen gradient as a consequence of the presence of the hemoglobin in the blood (Nahmias *et al.* 2006; Kidambi *et al.* 2009) and thus low oxygen concentrations do not mimic the natural hepatic environment (Cho *et al.* 2007), so cells oxygenation is a controversial issue.

On the following subsection it will be addressed the more relevant hepatic culture models. **Figure 1.5** depicts regular hepatocyte cultures in 2D.

A
Primary culture of
Human Hepatocytes



B
HepG2 cell line



C
HepaRG cell line

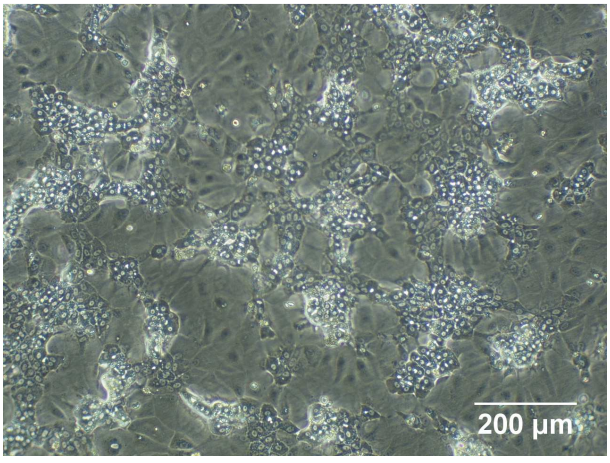


Figure. 1.5. Hepatocyte cultures in 2D. A – Primary culture of Human Hepatocytes; B- HepG2; C – HepaRG.

3.1. Human Hepatocytes

In the last few years, freshly isolated hepatocytes (**Fig. 1.5 A**) have been recognized as one of the most relevant models to study drug metabolism and transporter interactions, since this is the culture model that can best retain liver functionalities *in vitro* (Gomez-Lechon *et al.* 2010). Considered the golden standard of hepatic cultures, the use of freshly isolated hepatocytes is recommended both by the US Food and Drug Administration (FDA) and the European Medines Agency (EMA) for *in vitro* liver-related studies, such as induction determination.

Several studies with human hepatocytes have shown that, within the proper environment, human hepatocytes can retain the biotransformation mechanisms in an integrated form, including transporters and induction (Silva *et al.* 1999; Courtois *et al.* 2002; Donato *et al.* 2010; Rotroff *et al.* 2010; Perry *et al.* 2011), reproducing the *in vivo* paths and constituting a valuable tool to anticipate potential drug-induced liver toxicity. Thus, human hepatocytes have been used for hepatotoxicity studies, namely for: screening cytotoxicity and genotoxicity; characterizing drug-induced lesions, describing toxic mechanisms and determining biotransformation pathways (Coecke *et al.* 1999; Gomez-Lechon *et al.* 2010). However, liver-specific markers are hardly maintained in primary cultures and not at all after the maintenance of isolated human hepatocytes for several weeks. After isolation, hepatocytes undergo a natural de-differentiation that is characterized by the declining of protein synthesis, leading to a drop in the overall protein content and finally to cell death (Moshage *et al.* 1988).

To overcome de-differentiation several strategies have been developed to improve and prolong the human hepatocytes functionalities, these have focused on trying to reproduce *in vitro* a more physiological environment. For instances, human hepatocytes have shown a good adaptation and improved functionality when cultured in bioreactors (Schmelzer *et al.* 2009; Prot *et al.* 2011; Tostões 2011).

Another characteristic of these cells is the high donor-variability. In spite of being a limitation in terms of the reproducibility of some studies, is not really a disadvantage since it reflects the *in vivo* inter-individual differences. Moreover, this

model has shown robustness as it was possible to proceed to inter-laboratory validation. Reproducible results were obtained in different laboratories with harmonized protocols even for different donor cells (Richert *et al.* 2010).

As a way to further improve the human hepatocyte model, significant work on improving the efficiency of freezing and platting after thawing techniques has been done (Silva *et al.* 1999; Hewitt *et al.* 2007). **Cryohepatocytes** are freshly isolated hepatocytes that are maintained at -80ºC until use and have shown to perform similarly to the freshly isolated hepatocytes (Li *et al.* 1999; Li 2007). This has the advantage of having the possibility of using cells from same batch at different times. After thawing, they present lower cell viability, but the cells that are viable and able to attach, can perform similarly to fresh hepatocytes in spite of showing lower basal activities (Abadie-Viollon *et al.* 2010).

Human hepatocytes can be obtained from different sources such as livers unsuitable for transplantation, surgical waste material (after reduced-size or split-liver transplantation) and waste material from partial hepatectomy (Richert *et al.* 2010). However, the human liver tissue is sparsely available, being necessary to develop and use alternative hepatic culture models.

3.2. Rat hepatocytes

Alternatively to human cells, different mamalian cells have been used for development of *in vitro* liver models and hepatotoxicity assays since they are easier to obtain, namely monkey (Silkworth *et al.* 2005), porcine (Custer and Mullon 1998; Hoebe *et al.* 2000; Kostrubsky *et al.* 2000), rat (Guyomard *et al.* 1996; Shen *et al.* 2008; Hrach *et al.* 2011; Schutte *et al.* 2011) and mouse hepatocytes (Kulkarni and Khanna 2006; Jemnitz *et al.* 2008; Jaeschke *et al.* 2010).

Compared to the use of human cell lines, using animal freshly isolated hepatocytes has the advantage of better reflecting the *in vivo* hepatic features, having a more active and reliable metabolism. Within the different animal species it is difficult to choose the one that would best predict the human drug interactions.

Currently, the selection of a specie for toxicological assays is mostly based on the knowledge and historical use of that specie, the costs, the amount of hepatocytes obtained per each individual and the availability (O'Brien 2004), evaluating these parameters makes rat hepatocytes perfect candidates.

Primary cultures of rat hepatocytes are a good alternative often used by the pharmaceutical industry because: (i) they present higher metabolic responses than the common human cell lines and (ii) the inter-donor variability can be minimized by selecting animals of the same sex, age and similar feeding regimes (Coecke *et al.* 2000; O'Brien *et al.* 2004).

Rat hepatocytes, have shown good hepatocyte-specific characteristics such as cell shape and arrangement (Abu-Absi *et al.* 2002), biotransformation activities, (Kocarek *et al.* 1990; O'Brien *et al.* 2004) albumin secretion (Feldhoff *et al.* 1977), hormonal regulation (Coecke *et al.* 2000) and bilary transporters (Nakanishi *et al.* 2011).

In spite of the interspecies differences between rat and human, rat hepatocytes can be a good tool to explore culture strategies and undisclosed toxic cellular mechanisms. In the specific case of induction studies, although the differences in the sequences of the ligand domain of the nuclear receptor genes and the CYP lead to different induction responses (Lin 2006), the obtained information allows to interpret *in vivo* rodent results and thus conduct to the Refinement and Reducing of animal testing.

Moreover, the strategies developed to improve hepatocytes functionalities can often be easily applied to primary human hepatocytes. These extrapolations have shown successful results on the use of scaffolds (Bierwolf *et al.* 2010), 3D strategies (Brophy *et al.* 2009; Nakanishi *et al.* 2011), bioreaction (Allen *et al.* 2005; Miranda *et al.* 2009; Nakao *et al.* 2011), oxygen requirements (Kietzmann and Jungermann 1997; Nahmias *et al.* 2006; Cho *et al.* 2007; Kidambi *et al.* 2009) stem differentiation (Qihao *et al.* 2007) and co-culture (Coecke *et al.* 2000; Tilles *et al.* 2001).

In summary, the values obtained on rat hepatocytes are not physiological relevant, but the strategies adopted, and the methods developed have a good

correlation with what happens in human hepatocytes and with what happens *in vivo* (Richert *et al.* 2009).

3.3. Human Hepatic Cell lines

The main advantage on the use of hepatocyte cell lines, in comparison with freshly isolated hepatocytes is the easiness to culture: (i) is possible to freeze and thaw the cells with no further damage, (ii) cell karyotype can be maintain and (iii) have almost unlimited growth.

HepG2 is the hepatic cell line being used for a longer period of time, is a popular and good human cell line used to study a broad variety of toxicity mechanisms (Farkas and Tannenbaum 2005). The cells can be unlimited expanded while conserving the polyhedric hepatocyte-specific morphology *in vitro* (**Fig. 1.5 B**). In spite of being good models for toxicity accessment, on metabolism studies, like CYP induction, these cells have shown low metabolic competency (Farkas and Tannenbaum 2005).

After HepG2, several hepatic cell lines (HLE, THLE, BC2, Hep 3B) with increased metabolic competencies were developed. For instances, the recently developed cell line – Fa2N-4 – has shown promising results on its application for CYP3A4 induction during Drug-Drug interactions (Ripp *et al.* 2006). In spite of showing punctual relevant competencies, the major limitation of hepatic cell lines is that they do not show an overall metabolic realistic profile thus being of little value for integrative studies.

In 2002, a promising cell line called HepaRG was developed by Gripon *et al* which performed similar to human hepatocytes (Gripon *et al.* 2002). HepaRG is an hepatocarcinoma cell line with limited caryotype alterations, characterized by a surnumerary and remodeled chromosome 7 (Gripon *et al.* 2002).

After 4 weeks of differentiation including treatment with 2% DMSO, HepaRG generate two liver cell types: one resembling hepatocytes and the other resembling biliary canaliculi-like structures (**Fig. 1.5 C**). After detachment both structures can trans-differentiate into progenitor cells, which are able to differentiate again (Cerec et

al. 2007). During the last years, intensive characterization of HepaRG cells has shown that when differentiated these cells express the major cytochrome P450 (CYP), drug-conjugating enzymes, transporter proteins, nuclear receptors and transcription factors as well as other liver-specific proteins at levels close to those found in primary hepatocytes (Aninat *et al.* 2006; Guillouzo *et al.* 2007; Kanebratt and Andersson 2008; Turpeinen *et al.* 2009; Lubberstedt *et al.* 2011). These characteristics make HepaRG a good hepatic model for hepatotoxicity studies (McGill *et al.* 2011), CYP induction studies (Lambert *et al.* 2009; Andersson 2010), clearance (Zanelli *et al.* 2011) and liver pathological situations (Gripon *et al.* 2002; Schulze *et al.* 2011).

Moreover, this cell line has also shown good competency on an upper level of the development of 3R's ideology, by being compatible with more physiological models such as bioreactors (Darnell *et al.* 2011; Hoekstra *et al.* 2011) and even *in vivo* engraftment (Jiang *et al.* 2010).

3.4. Co-Cultures

For studies of metabolism, transporters as well as in hepatoxicity assays, monocultures of hepatocytes are usually preferred to mixed cultures. However, this scenario does not reflect the *in vivo* environment and the results can be misleading mainly because of the low hepatic performance *in vitro*, but also due to the lack of other cells that can affect the drug interaction with the hepatocyte as it occurs *in vivo*.

Regarding hepatic functionality, different studies have shown that cell-cell interactions, especially heterotypic cell interactions (Bhatia et al., 1998), are more effective in maintaining hepatocyte functions than ECM configurations (Michalopoulos *et al.* 1979; Khetani and Bhatia 2008). Significant enhancements in hepatocytes phenotype maintenance and function were described by the use of feeder cells in co-cultures with hepatocytes such as connective tissue or nonparenchymal cells in several species (Bhatia *et al.* 1998; Bhandari *et al.* 2001; Lu *et al.* 2005; Khetani and Bhatia 2008). Feeder cells are able to either secrete soluble signaling molecules (e.g. cytokines, hormones and growth factors) or provide other

cell-associated signals (i.e., insoluble extracellular matrix or membrane bound proteins).

The nonparenchymal cells used in co-culture with hepatocytes are usually endothelial cells, Kupfer cells, biliary cells but mostly fibroblasts. Fibroblasts are the most common cell type of connective tissue with a crucial role in the synthesis of extracellular matrix for tissue remodeling and repair. Additionally, they are the main producers of hepatocyte growth factor (HGF) (Stoker *et al.* 1987), a molecule involved in the anti-apoptotic mechanisms of hepatocytes (Hiramatsu *et al.* 2005), and since they have no ability of xenobiotic biotransformation their metabolism does not interfere with the toxicological assays (Donato *et al.* 1990).

Moreover, fibroblasts can increase the cell adherence both in 2D (Hui and Bhatia 2007; Evenou *et al.* 2011) and 3D cultures (Qihao *et al.* 2007) which improves cell stability, polarization and cell-cell contact.

The beneficial effects of feeder cells on hepatic performance was already validated *in vivo*, in studies where the engraftment of hepatocytes in co-culture with fibroblasts has shown to improve their secretory functionalities (Chen *et al.* 2011).

Additionally, co-cultures can also be a good model to study the cross-talk between different cell types. There are some successful studies performed with the co-culture of adipocytemyocyte/neuron/hepatocyte to study adipokines function. Recently Du *et al*., by co-culturing hepatocytes with epididymal and iguinal white adipose tissues have shown the depot-dependent effects of adipose tissue explants on the co-cultured hepatocytes in relation to the development of hepatic insulin resistance and cytotoxicity (Du *et al.* 2011).

4. HEPATOTOXICITY

The major cause of a compound failure in the Drug Development process is drug hepatotoxicity in the clinical phase (Ostapowicz *et al.* 2002), often due to the lack of knowledge about the toxic process. A bioactivated drug can either have a pharmacological or a toxicological effect in the liver and, in fact, most liver injuries are caused by bioactivated compounds (Gomez-Lechon *et al.* 2010). Due to its high vascularization and biotransformation function, the liver is the first target of drug toxic effects causing different type of lesions that can be classified in: i) zonal hepatocellular alterations without inflammatory reaction, ii) intra-hepatic cholestasis (including cirrosis), iii) hepatic necrosis with inflammatory reaction or iv) hepatocarcinoma (Casarett and Doull 1991). However the toxic mechanism of drugs is still not fully understood, and Drug-induced liver injury (DILI) diseases still represent a major challenge for clinicians, the pharmaceutical industry and regulatory agencies worldwide, including EMA (EMA 2010) and the FDA (Kaplowitz 2005).

Inside the cell, hepatoxicity can be the consequence of chemical reactions such as covalent binding, oxidative stress, lipid peroxidation, alteration of Ca^{2+} homeostasis and mitochondrial injury that will lead to apoptosis or necrosis (Gomez-Lechon *et al.* 2010). For a better understanding of the hepatotoxicity events, it is important to use *in vitro* detection methods that allow to follow the different cellular events such as decreasing of viability (*MTT, XTT, Neutral Red, Alamar Blue)*, cell death (leakage of lactate dehydrogenage and alanine amino transferase), cell damage (DNA damage, protein adducts, lipid peroxidation, morphological alterations and changes in drug metabolism, gene expression and protein expression) and also use appropriate methods to access hepatocyte-specific metabolisms (i.e. gluconeogenesis, ureogenesis, plasma protein synthesis, GSH, NADH and ATP levels) (Kaplowitz 2005; Gomez-Lechon *et al.* 2010).

Having such diverse possibilities of drug effects, it is important to have *in vitro* cell culture strategies that allow simultaneous screening of conditions. In the last years the high-throughput concept has been largely developed and implemented,

and thus it is already included in the new technologies for drug toxicity applications (Weyermann *et al.* 2005; Nakanishi *et al.* 2011) and is part of the basic requirements of *in vitro* liver models for drug screening in industrial scale (Sundberg 2000; Zhang *et al.* 2011).

5. *IN VITRO* 3D MODELS IN TOXICOLOGY

Most of *in vitro* cell assays/tests are commonly performed in the two-dimensional (2D) cultures assuming that this will reflect the *in vivo* response. In general, this type of cultures consists of a monolayer of cells attached to a rigid surface that do not represent the essential physiology and architecture of the real tissues. In the last years the use of cell culture strategies that could better represent the physiological cell environment has been the focus of many research groups that aim at achieving structures presenting more cell-cell contacts, the so called three dimensional (3D) strategies. In the last two decades, studies have, in fact, shown that 3D cultures can reduce the gap between cell cultures and physiological tissue. In addition to increasing the cell-cell contact, 3D structures induce the cells to secrete extracellular matrix (ECM) proteins which results in a gel-like stiffness geometry, and specific *in vivo* biochemistry (Evenou *et al.* 2011) that is impossible to obtain with the 2D structures.

Considering their improved features, 3D cultures have been applied to the reconstitution of many types of tissues and structures, such as different types of tumours, skin tests, osteogenesis studies, absorption assessment and in tissues such as cornea, brain, heart, lung, kidney and liver (Pampaloni *et al.* 2007; Santos *et al.* 2007; Gurkan *et al.* 2011; Jung *et al.* 2011; Nyga *et al.* 2011; Shamis *et al.* 2011; Wu *et al.* 2011). All together, the availability of the several approaches and the results brought up by 3D strategies lead the scientific community to believe that they will have a crucial role on the replacement of animals for drug testing (Bhogal *et al.* 2005; Pampaloni *et al.* 2007; Pelkonen 2010).

The complexity of the validation process concerning both time and money, has only allowed the validation of a short number of 3D models (ECVAM, http://ecvam.jrc.it). Nevertheless, some 3D models are already fully validated, namely models for skin (EPISKIN™, Epiderm™ and SKinEthic™) (Bhogal *et al.* 2005; Netzlaff *et al.* 2005), Corneal epithelium, Tracheal/bronchial epithelium, vaginal cervical and for bucal and gingival mucosa (MakTek, SkinEthic) (Pampaloni *et al.* 2009).

Moreover, in order to have 3D models representing other tissue types, that fullfill the previous metionted requirments for validation (sub-Chapter 1.1.) there are still missing a few features including accurate compatible methods regarding systemic sample collection, innovative methods for biochemical analyses, improvement and standardization of 3D cell culture protocols and new quality control parameters (Pampaloni *et al.* 2007; Mazzoleni *et al.* 2009).

Table 1.3 summarizes the 3D strategies currently used or with potential application for toxicological assays. Despite the advantages of 3D approaches, all the current strategies still present some limitations highlighting the need of further development of these approaches.

Table 1.3: 3D Models available (Adapted from (Mazzoleni *et al.* 2009))

System		Advantages	Disadvantages
Tissue slices / Tissue explants		- Preserve part of tissue architecture and cellular interactions - Maintain tissue-specific functions for longer time than perfused organ	- Cell viability and differentiated phenotype are limited to few days
Scaffold- / Microcarriers-based cultures	***Naturally derived materials*** (e.g. collagen or fibrin) - ***Synthetic polymers*** [e.g. poly(dimethylsiloxan), poly(DL-lactide-coglycolide), poly(glycerolsebacate)]	- Engineered scaffolds provide physical/structural/ biochemical support - Sustain cell viability and tissue-like functions - Can be used in all dynamic/ perfused culture systems	- Spatial variations in nutrients, oxygen, metabolite concentrations may exist and modify cell behaviour randomly throughout the scaffold
Organotypic cultures	***Multicellular spheroids***	- Reconstitution of tissue-like organisation (polarity, function, viability) - Scaffolds can be avoided	Cell life span may vary, depending on the type of bioreactor
Gel- / Matrix-based cultures	***Hydrogels (ECM like):*** *Natural product-based hydrogels* (e.g. collagen, fibrin, Matrigel) *Synthetic selfassembling peptide hydrogels*	Differentiated phenotype can be maintained for several days, depending on the cell type	Harvesting of cells could be optimised - Problems of mass transfer if not coupled to perfused systems
	3-D surfaces (BM like): *Synthetic* (fixed combination of various ECM components) *Cell-/ tissue-derived Biomatrices*	Situation more similar to *in vivo* conditions - Sustain cell viability, polarity, function	Composition and structure of matrices may vary between preparations - Standardised protocols are needed

(Table 1.3. cont.)

System		Advantages	Disadvantages
Dynamic systems	- Roller bottles - Gyratory shakers - Spinner flasks	- Moderate to high mass transfer to provide nutrients and to export wastes - Increase cell viability and allow longterm studies	**- Intermediate to high shear stress**
Rotary cell culture systems	Rotary Cell Culture Systems (RCCS)	- Low shear forces and turbulence - High mass transfer - Maintain and/or favour tissue-like organisation, polarity, function - Increase cell viability	**- Expensive**
	Rotary Perfusion RCMW	- Perfusion extends RCCS potential - Available as multi-compartmental devices	**- Difficult to handle for screening test purposes** **- Expensive**
Perfused models	Hollow-fiber perfused systems - Airlift bioreactors - Direct perfusion bioreactors - Packed-bed bioreactors	Controllable and reproducible microenvironment - Low to intermediate shear stress - Efficient mass transfer - Long-term maintenance of tissue-like functions and cell viability - Work with multi-compartmental devices allowed	**- Limited 3-D cell growth** **- Cost more or less significant, depending on bioreactor type**
Microfluidic systems	Microfluidic systems - Microfluidic biochips	See Perfused models - Nutrient / fluid gradient possible - Lab on chip (on-line investigations)	**- Limited 3D cell growth**

5.1. 3D Liver Systems

The rapid de-differentiation of cultured hepatocytes together with the low metabolic profile of hepatic cell constitute the major limitations of *in vitro* hepatic approaches. Thus, in the last years, the reconstructing of the spatial liver re-arrangement using 3D strategies has been adopted as a strategy to improve the recreation of liver-specific functions *in vitro.*

Moreover, 3D liver systems have been used to improve models associated with some aspects of drug discovery and transport studies. Using different approaches it has been shown that 3D strategies lead to a specific or global improvement and extend of hepatic activities and their use has already been successfully applied to toxicity studies (Schutte *et al.* 2011). The functional improvement of hepatocytes observed in 3D cultures results first of all from the cell organization. More specifically, studies which profiled the integrin expression of primary hepatocytes *in vitro*, suggest a dependence on juxtacrine cell interactions in 3D for the maintenance of important functions such as biotranformation and albumin synthesis (Liu Tsang *et al.* 2007). Furthermore, the 3D arrangement enables cell polarization as it occurs *in vivo* (**Fig. 1.3 A**), allowing a better function of the transporters and the phosphoralization of receptors such as Hepatocyte Growth Factor (HGF) and Epidermal Growth Factor receptors which are important factors for hepatocellular differentiation and activation (Engl *et al.* 2004).

A broad range of 3D cell culture techniques have been employed to develop liver models, in this sub-section the most significant strategies used over the last years to perform 3D hepatic cultures will be described.

5.1.1. Liver slices

The use of liver slices for *in vitro* hepatic studies has been used since 1923 (Ekins 1996), and is still currently the approach more representative of the *in vivo* structure (**Fig. 1.6 A**). Slices have two-cut surfaces with a thickness greater than 100 μm to maintain a significant ratio of undamaged to damaged cells (Vickers and

Fisher 2004). The use of liver slices has several advantages, such as being from an human source, keeping the *in vivo* structural arrangement and polarization (since no proteolytic enzymes are used), have high cell-cell contact and the presence of other cells (Vickers and Fisher 2004) and thus it is considered a good *in vitro* model. These characteristics have shown to maintain organelle integrity, and cellular functions such as induction of CYP2B, CYP3A, CYP1A, CYP2A5 (Glockner and Muller 1995; Lake *et al.* 1996; Gokhale *et al.* 1997; Lake *et al.* 1997). However, the performance accuracy of the studies always relies on the availability of human livers and specially on the quality of the tissue used (Vickers and Fisher 2004). Furthermore, culture oxygenation and nutrient diffusion from the culture medium are not enough to maintain viable and active cells longer then 24-72h (Engl *et al.* 2004; Farkas and Tannenbaum 2005).

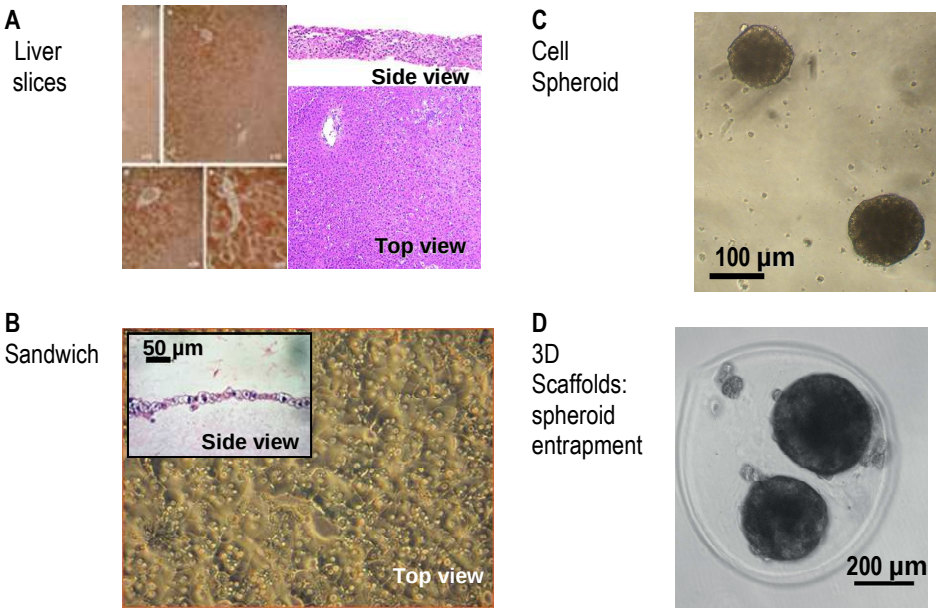


Figure. 1.6: 3D approaches for hepatocyte cultures A – Liver perfused slices (Adapted from (Vickers *et al.* 2004; Alino *et al.* 2007)); B – Human hepatocytes in Sandwich cultures (adapted from (Kostrubsky *et al.* 2003) and http://www-alt.igb.fraunhofer.de/www/gf/tissueengineering/gewebemodelle/en_/liver-models.en.html); C – cell spheroids; D – gel entrapped cells (Adapted from (Tostões *et al.* 2011)).

5.1.2. Sandwich

The actual Sandwich strategy consists of culturing hepatocytes in between two layers of gelled extracellular matrix proteins, including collagen I and matrigel proteins (Hewitt *et al.* 2007) (**Fig. 1.6 B**). The first approach to this strategy was performed by Dunn and his co-workers in 1989 by culturing rat hepatocytes in the middle of two collagen layers (Dunn *et al.* 1989). This was the first 3D strategy that allowed prolonging the longevity of hepatic-functions in culture while maintaining the cell polarity.

Recently, this strategy has been further improved and adapted to include among other features, oxygen control (Bader *et al.* 1999) and high-throughput studies (Zhang *et al.* 2011). In addition to metabolic studies, this strategy has also been applied to hepatocyte differentiation studies (Novik *et al.* 2008) as well as to *in vitro* model of excretion (Liu *et al.* 1999).

In spite of all these factors, the use of collagen in culture brings several limitations to the culture. This gelatin matrix is from animal origin, bringing different species factors to the culture, with a high batch to batch variation (Kim and Mooney 1998). Furthermore, there are also other drawbacks to its application to drug testing, namely it is observed that the mass transfer through the collagen matrix is poor which is accompanied by variable drug absorptions (Du *et al.* 2008).

5.1.3. Cell Spheroids

Hepatocyte spheroids (**Fig. 1.6 C**) are the best characterized 3D model with a wider range of applications as result of its simplicity, reproducibility and similarity to the *in vivo* system (Tung *et al.* 2010). The 3D hepatic spheroid is usually characterized by a smooth surface containing pore like openings that help on diffusion and can, in many cases, resemble the bile canaliculi (van Zijl and Mikulits 2010).

There are multiple possibilities of culturing 3D hepatospheres such as: spontaneous spheroid formation in non-adherent Petri dishes or well plates (Gurkan

et al. 2011); matrices/micropattern which facilitates the formation of spheroids (Lee *et al.* 2008; Okuyama *et al.* 2010; Fukuda and Nakazawa 2011) (cells are seeded on non-adhesive surfaces, such as 3D alginate porous scaffolds, where small amounts of cell suspension are incubated upside down until spontaneous spheroid formation occurs – hanging drops) (Tung *et al.* 2010); or even through the application of mechanical forces such as the rocking technique, employing a rotating wall vessel (Pampaloni *et al.* 2007; Brophy *et al.* 2009; Ishikawa *et al.* 2011) or even using stirred tanks (spinner vessels) (Abu-Absi *et al.* 2002; Miranda *et al.* 2009; Leite *et al.* 2011; Tostões *et al.* 2011).

In all the described cases, the common characteristic is the possibility to prolonge the life-span of hepatocytes by increasing their specific functionalities. Moreover, studies have demonstrated that hepatospheres can increase the genetic expression of both the xenobiotic metabolism and the lipid metabolism by increasing the up-regulation of leukotrine and cholesterol metabolism and of the synthesis of glutathione, albumin and ATP. In addition, hepatocytes cultured as 3D spheroids present an active urea cycle and express liver-enriched transcription factors (van Zijl and Mikulits 2010).

In summary, spheroid cultures have been shown to be a promising physiological structure for *in vitro* toxicity assessment, to investigate the impact of drugs in the liver, but also for tissue engineering. The limitations of the 3D spheroids are dependent on the apparatus use to generate them, some of those limitations are described in the bioreactor section and also on **Table 1.3**.

5.1.4. 3D Scaffolds

Hepatocyte entrapped cells have been used in a diverse array of studies such as toxicity studies (Meng *et al.* 2007; Shen *et al.* 2008), cryopreservation (Guyomard *et al.* 1996; Rialland *et al.* 2000) and cell therapy applications (Clement *et al.* 1998;

Haque *et al.* 2005) showing successful results in terms of cell activity or protection from physical forces.

The use of hydrogel matrices intends somehow to mimic the *in vivo* extracellular matrix (ECM) creating a more physiological stiffness. Providing a cell ECM will generate a microenvironment where the cells will be protected from external damages and the provided structural support will help to generate a 3D structure (**Fig. 6 D**). Besides the physical properties, these structures allow the ECM-cell contact providing the cells with signaling moieties, proteins and growth factors present either on the gel molecular composition or immobilized within it. Moreover the entrapment of cells in gelic scaffolds allows the application of dynamic/perfused culture systems without cell damage (Mazzoleni *et al.* 2009).

Currently, the selection of hydrogel scaffold methods is largely based on material availability, nonetheless in the case of hepatocyte cultures, Collagen, Matrigel and Alginate are preferably used. Properties like biodegradability and biocompatibility are a major concern due to the need to immobilize hepatocyte aggregates for tissue engineering and to generate bioartificial organs.

Substantial efforts have been done to develop these applications; however there are still some limitations common in the use of hydrogels. The scaffold can modify randomly the cell behavior and dead cells might remain entrapped within the gel signaling the neighbor cells with necrotic factors. In addition to the already mentioned diffusion limitations and the unwelcome interactions within the scaffold, the gel can also induce variations on the nutrient, oxygen and metabolite concentrations (Mazzoleni *et al.* 2009).

5.2 Bioreactor Systems

Mammalian tissues, due to their specific nutrient needs, sensitivity to nitrogenated wastes and their fragility to sheer stress have in general several limitations to their *in vitro* maintenance (Mazzoleni *et al.* 2009). Biotechnology developments are leading to bioreactors that closer and closer mimick the *in vivo* cell

environment. From the first approach, using orbital shaken Petri dishes, to the current state-of-the-art bioreactors have exponentially progress in terms of oxygen control, nutrient supply, amenable and well characterized environment. The concept of dynamic culture conditions has been introduced allowing for improved mass transport, especially in 3D cell structures.

Many parameters have to be taken into consideration in order to develop an accurate system that mimics the *in vivo* milieu by adapting the necessary thermodynamic and environmental parameters. The specific surroundings of each tissue within the body require different optimal *in vitro* cell environments, which are obtained through the control and monitoring of the bioreactor settings such as flow, temperature, pH and oxygen, according to the cell's demands.

Regarding hepatocyte cultures, oxygen is a special sensitive parameter as it was already described in sub-Chapter 1.3. Hepatocellular oxygen uptake rates in culture are limited by the medium thickness as well as its surrounding oxygen concentration. In spite of the fact that hepatocytes can tolerate hypoxia, since they can satisfy their ATP levels by anaerobic glycolysis (Bader *et al.* 1999), this is nonetheless a non physiological approach.

Having limiting levels of oxygen in culture will affect hepatocyte polarization (Kidambi *et al.* 2009) and enzymatic performance, specially moonoxygenases reactions (phase I CYP 450) (Jungermann and Kietzmann 1997). On the other hand, supraphysiological oxygen concentrations lead to the formation of free radicals which in turns results in cell injury (Stevens 1965). The perfect balance can be obtained by using oxygen carries (Nahmias *et al.* 2006) or by culturing the cells in bioreactors since these allow to measure and control the oxygen levels in the medium, in addition bioreactors flow system helps diffusion both in the culture medium and in the cells.

Furthermore, the possibility to impose different oxygenations can mimic pathological situations (such as tumours) as well as the perivenous (3-5%) and periportal oxygenations (8-9%) (Jungermann and Kietzmann 1997) known to

modulate the zonal gene expression and affect drug metabolism and toxicity (Allen *et al.* 2005; Yan *et al.* 2010).

In toxicological studies it is important to have a system that does not imply the use of large amounts of compounds, but that at the same time can be expanded generating large amounts of cells in the same unique microenvironment allowing it to be compatible with screening tools that enable the parallelization of biological assays thus, facilitating better diagnostics and improving functionality.

Over the last years, several bioreactors that allow the monitoring and control of the above described parameters have been develop with the aim of culturing hepatocytes as 3D cultures. These include hollowfiber systems (De Bartolo *et al.* 2000), low shear perfusion systems (Chen 1992), stirred tank bioreactors (Reuss 1995), airlift bioreactors (Wang *et al.* 2005) and rotary cell culture reactors (Saxena *et al.* 2007) among others. In this section the most relevant bioreactors currently being used for 3D hepatocyte culturing are discussed (**Fig. 1.7**).

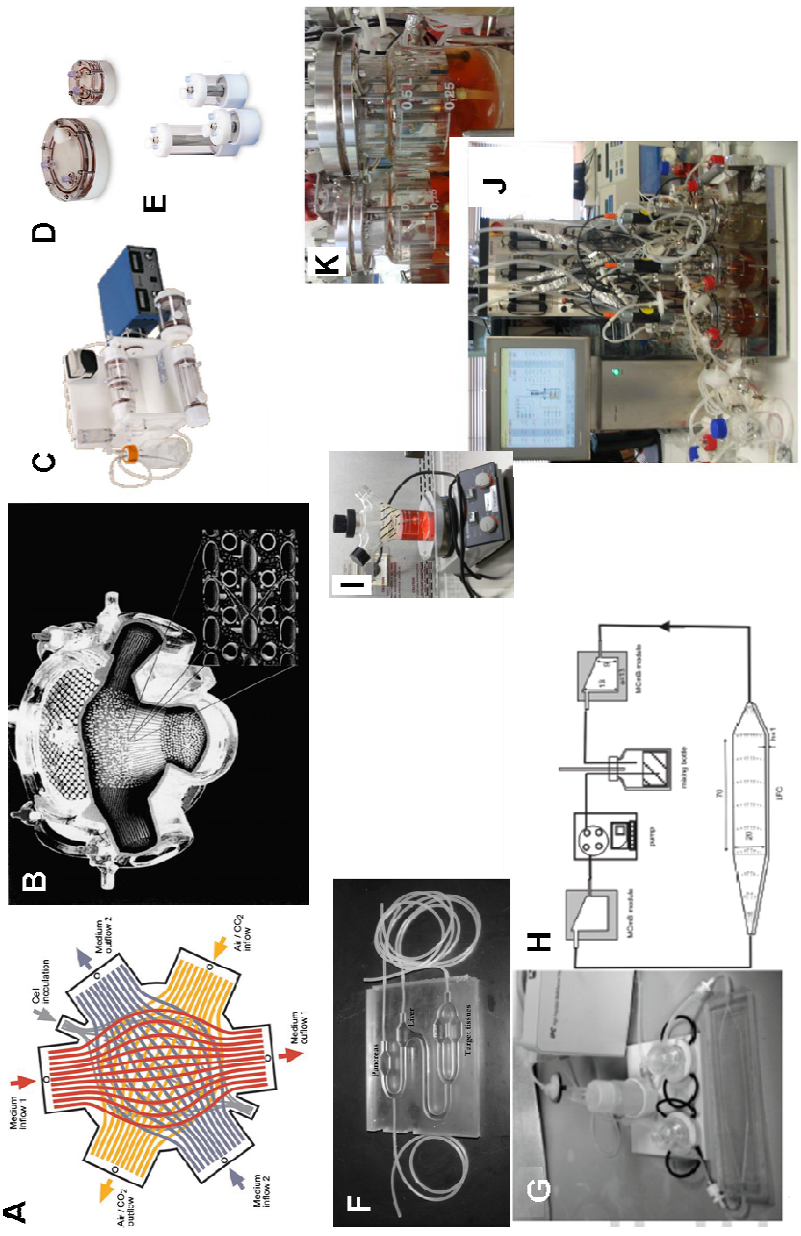


Figure 1.7: Bioreactor systems for hepatocyte cultures. (A-B) Multicompartment 3D perfusion hollow-fiber bioreactor (A,B) (adapted from (Gerlach *et al.* 2003) and (Schmelzer *et al.* 2009)); (C-E) Roratory Wall vessel Bioreactor (www.synthecon.com); (F-H) Microfluidic/Multicompartmental bioreactors (adapted from (Vozzi *et al.* 2011) and (Vinci *et al.* 2011)); (I-K) Stirred Bioreactors: (I) Spinner vessel; (J-K) Fully controlled stirred tank bioreactor (BIOSTAT® Qplus): (J) bioreactor unit, (K) stirred vessels.

5.2.1. Multi-compartment 3D perfusion bioreactors

The perfused hollowfiber bioreactor developed by Gerlach *et al* (**Fig. 1.7 A,B**) (Gerlach *et al.* 1994) was designed based on the vascularized structure of the liver, having a synosoid like structure, with inlet and outlet of medium resembling the blood flow. The technology is based on the use of interwoven hollow fiber capillary membranes that provide independent, decentralized medium and gas supply to the cells located between the capillaries. When cultured in this type of bioreactors, porcine and human liver cells have shown to retain *in vivo* like properties and are arranged in tissue-like structures including the formation of biliary canalicular networks and neo-sinusoids (Zeilinger *et al.* 2004; Schmelzer *et al.* 2009), maintaining liver specific function such as urea and albumin synthesis, glucose metabolism and CYP activities. Later, similar results were obtained when culturing the hepatoblastoma cell line HepaRG (Darnell *et al.* 2011). Furthermore, this technology has been developed not only for toxicological applications but also as an extracorporeal liver support system, as *in vitro* liver cell cultures provide an option for bridging the organ functions until regeneration occurs or until transplantation can be performed (Schmelzer *et al.* 2009). Depending on the application, it is possible to use different sizes of the hollow-fiber bioreactor, being possible to minimize the use of expensive compounds using small volume bioreactors (Zeilinger *et al.* 2011).

In spite of being a very good strategy for culturing hepatocytes, this system has the disadvantage of not allowing cell sampling/recover, for further analysis. Moreover, the possibility of polyethersulphone capillars influencing the solubility of the drug test compounds in the medium cannot be clearly excluded.

5.2.2. Rotational Wall Vessel (RWV)

The Rotating Wall Vessel (RWV) bioreactors developed by N.A.S.A.'s Johnson Space Center technological research in USA (http://science.nasa.gov/NEWHOME/br/bioreactor.htm) consists of an horizontally rotating, transparent

clinostats that leave no head space between atmosphere and culture medium (**Fig. 1.7 C-E**). The rotation allows the formation of 3D spheroid structures that will improve the cell-cell contacts yielding a higher gene expression of several cell types (Mazzoleni *et al.* 2009). Since this system does not have internal moving parts, shear forces and turbulence normally associated with impeller-driven stirred bioreactors, are reduced to a minimum. The vessel design and motion avoids cell sedimentation and inadequate gas/nutrients supply guaranteeing the most favorable conditions for cell/tissue culturing (Mazzoleni *et al.* 2009). In general, the main relevant characteristic of RWV is to ensure a good ratio of low shear environment to high mass transfer.

When applied to toxicological studies, the main limitation of RTW together with the fact of being an expensive technology is the impossibility to use for screening test purposes.

5.2.3. Microfluidic / Multicompartmental bioreactors

The Microfluidic or Multicompartmental bioreactors (MCmB) (**Fig. 1.7F-H**) are modular devices where different cell types are cultured in separated chambers but are connected by medium flow. In spite of their main common characteristics there are different types of microfluidic bioreactors that slightly differ from each other being possible to have microfabricated devices such as perfused microarray bioreactors (Powers *et al.* 2002), micro cell culture chambers (Lee *et al.* 2006) or micro cell culture analogs (Sung and Shuler 2009). The cells within these devices can be entrapped in gels, organized as spheroids or attached to the wall of microchannels and microchambers located in the bioreactors.

The advantages of the flow systems are the continuous renewal of nutrients and oxygen as well as the facilitated waste removal, these simultaneously induce shear stress which helps the diffusion inside the cells stimulating both cell growth and function (Vozzi *et al.* 2011). Furthermore, the flow creates physiological like situations on the hepatic tissues, such as liver zonation or shear stress (Tilles *et al.*

2001; Allen *et al.* 2005), and allows to perform drug metabolism induction following chemical administration (Fukuda and Nakazawa 2011).

In a physiological perspective, this system resembles the human body. Studies carried on by Vinci *et al.* (Vinci *et al.* 2011) have shown that the culture of 3 types of cells in this system, namely adipose tissue, endothelial cells and hepatocyte cells, reproduces *in vivo* the regulatory characteristics act homeostatic-like regarding the control of glucose and in addition the metabolic cross-talking of hepatocytes leads to *in vitro* cell regulation.

These systems have the additional advantages of being highly flexible towards adaption and being compatible with high-throughput technologies. However, the low volume of these reactors limits the sampling and metabolites analyses, as well as cells recovery. Furthermore, surface and edge effects are amplified whereas physiological cell–cell interactions are under-represented (Mazzei *et al.* 2010; Vozzi *et al.* 2011).

The miniaturized characteristic of this bioreactors make them easy to work and cost effective regarding the use of culture medium and test compounds. However, they have inherent mass transfer limitations on the maintenance of 3D structures, only overcame by increasing the perfusion rates which implies a higher shear cell stress. Additionally, it is difficult to control parameters such as oxygen and pH in these microsystems.

5.2.4. Stirred culture vessels

Stirred culture vessels, including spinner vessels and environmental controled stirred bioreactors (**Fig. 1.7 I-K**), are hydrodynamically well described vessels with an inner impeller that generates convective forces provided by the stirrer and medium flows around the cells. This stirring, at the same time that allows spontaneous generation of 3D spheroids, helps the diffusion of nutrients and oxygen inside the cells and the removal of cell's waste products.

The main advantage of these bioreactors in comparison to the previously ones (**Fig. 1.7 A-H**) is the possibility of sampling and cell recovery during culture time. Cell

sampling will allow an on-line cell characterization and quantification but also the feeding of smaller systems, such as well-plates, with cells from a common bulk for screening tests.

Spinner vessels (**Fig. 1.7 I**) are very attractive approaches due to their simplicity and low costs, but on the other hand environmental control stirred bioreactors provide an automated control of the environment (**Fig. 1.7 J,K**), allowing the on-line monitoring and control of specific culture variables (temperature, pH, dissolved oxygen, nutrients) improving culture outcome and ensuring reproducibility. In the special case of oxygen, the sensitivity of hepatocytes to this parameter can be overcome by this type of bioreactors with internal probes and automatic gas injectors allowing the generation of a continuous oxygen supply avoiding the commun exposure of the cells to supraphysiological oxygen levels (Miranda *et al.* 2009; Leite *et al.* 2011; Tostões 2011; Tostões *et al.* 2011).

However, these culture systems also present some drawback such as the production of spheroids with variable diameters (Tostões 2011), the need for significant high volumes and inducing some shear stress on the cells of the outside layer of the spheroids with the padle stirring.

6. SCOPE OF THE THESIS

This thesis is focused on the development of 3D strategies for hepatocyte culturing using bioreactor devices towards the development of *in vitro* competent, highly predictable methods for application on Drug development. More specifically, the 3D spheroid approach was used to culture hepatocytes in stirred vessels, taking the described advantages to enhance hepatocyte-specific functions and extend its life span in culture, thus improving on the previously available models.

The overall goal was to obtain a more physiological, reproducible and robust approach that could be applied in drug testing for both acute and chronic toxicity. To achieve this, three relevant hepatic cultures were used:

i) Human Hepatocytes, cells freshly isolated from human liver. These retain better the liver specific activities and are considered

the golden standard of hepatic cultures. Aiming at prolonging human hepatocytes longevity by improving their metabolic profile a 3D strategy was used.

ii) Rat Hepatocytes, freshly isolated from the animal. Since Human hepatocytes are sparsely available, while developing and optimizing the system rat hepatocytes were used instead of human hepatoytes as model system.

iii) HepaRG cell line, was used as an alternative to the use of human hepatocytes. This human cell line has shown higher activity than other cell lines and could be an attractive alternative to the use of primary cultures, overcoming their limited availability problem.

Specifically, the strategy developed intendeds to improve the hepatocyte biotransformation profile. When cultured in the environmental controlled stirred bioreactor, the parameters were set close to the *in vivo* values. To address the biotransformation capacity phase I and phase II activities were assessed during culture time by measuring CYP and UGT specific activities respectively. Moreover, as a whole system characterization, it was also monitored the albumin secretion and ammonia detoxification by synthesis of urea. These values together with immunofluorescence characterization of the cell spheroids could give an overall image of the system in development.

Furthermore, to test the physiological response of the system, the 3D Cultures in stirred vessels were challenged by testing clearance, different oxygenations and toxicity, using specific reference compounds. The capacity of the cells to respond to induction was also evaluated as an important quality control of hepatic metabolism. Additionally, testing induction capacity of the hepatocytes, a complex cell path can be assessed by performing a simple assay.

A schematic representation of the main goal proposed for this thesis as well as the strategy that was employed to address it is summarized on **Figure 1.8**.

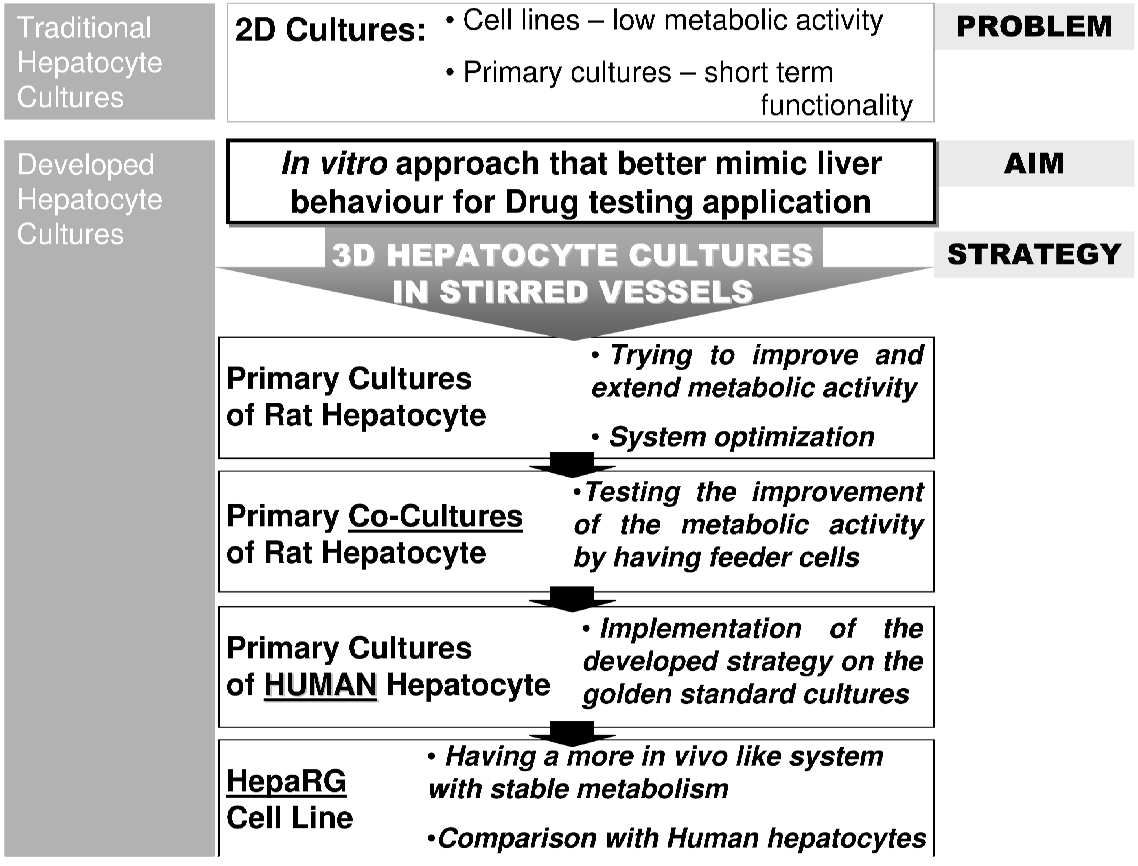


Figure. 1.8. Schematic representation of the aim and strategy of this PhD thesis.

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II

RAT HEPATOCYTES IN 3D

This chapter is based on the following manuscript:

Toward Extended Functional Hepatocyte *In Vitro* Culture
Joana P. Miranda, Sofia B. Leite, Ursula Muller-Vieira, Armanda Rodrigues, Manuel J.T. Carrondo, and Paula M. Alves (2009) Tissue Engineering: Part C, 15(2) (2009): 157-67.

Sofia B. Leite had carried out all the experimental part performed on the bioreactor, as well as involved on the decisions on how to execute the experiments, as well as on the discussion and interpretation of the results.

ABSTRACT

Primary cultures of human hepatocytes are a reference cellular model, because they maintain key features of liver cells *in vivo*, such as expression of drug-metabolizing enzymes, response to enzyme inducers, and generation of hepatic metabolites. However, there is a restricted availability of primary hepatocytes, and they show phenotypic instability in culture. Thus, different alternatives have been developed to overcome the culture limitations and to mimic *in vivo* tissue material. Herein, culture conditions, such as medium composition, impeller type, and cell inoculum concentration, were optimized in stirred culture vessels and applied to a three-dimensional (3D) bioreactor system. Cultures of rat hepatocytes as 3D structures on bioreactor, better resembling *in vivo* cellular organization, were compared to traditional monolayer cultures. Liver-specific functions, such as albumin and urea secretion, phase I and phase II enzyme activities, and the capacity to metabolize diphenhydramine and troglitazone, were measured over time. Hepatocyte functions were preserved for longer time in the 3D bioreactor than in the monolayer system. Moreover, rat hepatocytes grown in 3D system maintained the ability to metabolize such compounds, as well as *in vivo*. Our results indicate that hepatocytes cultured as 3D structures are a qualified model system to study hepatocyte drug metabolism over a long period of time. Moreover, these cultures can be used as feeding systems to obtain cells for other tests in a short time.

CONTENTS

1. INTRODUCTION

Primary cultures of hepatocytes constitute a powerful tool that has been extensively used in biomedical research both in academia and in bioindustry settings. In particular, hepatocyte cultures have been extremely useful in drug development. Liver damage caused by medications or chemical compounds is a key reason for the ever increasing and expensive phenomenon of drug recalls. Because the liver is the main organ for biotransformation and metabolism of numerous endogenous substances and pharmacological agents, various *in vitro* liver models, including isolated liver slices and perfused livers, primary cultures of hepatocytes, and immortalized hepatic cell lines in different cultivation formats, have been utilized to better understand drug response in humans (Fabre *et al.* 1990; Castell *et al.* 2006). Although cultures of human hepatocytes would be the ideal model to perform such studies, to overcome interspecies differences in all aspects of hepatic function, their source is limited because they can only be obtained from liver biopsies (Li *et al.* 1999; Grompe 2001). Rodent hepatocytes, on the other hand, have been successfully used in pharmacological and toxicological studies (Bort *et al.* 1999; Yan and Caldwell 2001; Jaeschke *et al.* 2002; Wang *et al.* 2002). These resources of hepatocytes have the advantage of being easy to access; the results drawn from these experiments are reproducible and consistent, and therefore optimal when it concerns to the development of new culture methods. Primary cultures of hepatocytes currently are the system that better mimics the organism. However, hepatocytes grown in culture and maintained under standard *in vitro* cell culture conditions are known to rapidly lose liver-specific functions, especially in biotransformation involving phase I and phase II enzymes (Skett *et al.* 1999). Therefore, a system where primary hepatocytes maintain *in vivo*–like hepatic-specific functions in culture for longer periods of time would powerfully contribute for drug metabolism research. Among many reasons that may account for a deficit of hepatocyte-like functions *in vitro*, cell adaptation to culture conditions after withdrawal from their organ environment seems to be one of the major problems. Several

strategies have been developed to overcome this loss of function, such as protein synthesis, cell membrane integrity, and maintenance of life span limit and of cytochrome P450 (CYP 450) activity (Li *et al.* 1999). Cultivation of hepatocytes on different extracellular matrices (Kern *et al.* 1997) and in different culture systems, or the supplementation of media with different hormones and growth factors was shown to improve *in vitro* hepatocyte quality (Kern *et al.* 1997; Nussler *et al.* 2001; Pfaller *et al.* 2001). Culturing hepatocytes as three-dimensional (3D) structures provide cells with an *in vivo*–like environment, enabling the retention of important hepatic functions (Dunn *et al.* 1989; Kern *et al.* 1997; Schmitmeier *et al.* 2006) without the need to add extracellular matrix components. Approaches such as gel encapsulating systems and spheroid cultures have dramatically improved our understanding of the role of 3D culture strategies upon hepatocyte functions, but *in vitro* and *in vivo* models under well-defined and reproducible conditions are still needed (Fischbach *et al.* 2007). To fully control a culture environment, one has to resort to bioreactors. Several bioreactors of bioartificial livers have already been applied in therapy (De Bartolo *et al.* 2000; Gerlach *et al.* 2003; Gerlach 2006; Son *et al.* 2006; Fiegel *et al.* 2008). Further, bioreactors for application in both pharmacological and toxicological studies have been developed (Brown *et al.* 2003; Schmitmeier *et al.* 2006). In our laboratory a system with a fully controlled environment, successfully applied to other cell types (Sa Santos *et al.* 2005), could be adapted to hepatocyte cultures bringing several advantages over the others. Besides providing a better control of cellular environment, it would give a more predictive response of *in vitro* cultures that would improve the economics of cell-based testing. Herein, the challenge was to establish a 3D *in vitro* model for primary rat hepatocytes consisting of a fully controlled minibioreactor that would allow for drug screening. The final result consists of a stirred tank with a controlled environment (temperature, pH, and pO2), which is reproducible, allows access to the cells (sampling), and is amenable to large-scale use. Besides, it could be easily adaptable to other types of cells, such as human hepatocytes or even human embryonic stem cells (hESC)-derived hepatocytes, and to be used as a feeding system for parallel cell tests.

2. MATERIALS AND METHODS

2.1. Rat hepatocyte isolation

Hepatocytes were isolated from male Wistar rats, 6–9 weeks old, with 200–300g body weight (from Instituto de Higiene e Medicina Tropical, Lisbon, Portugal, Animal House). A two-step collagenase perfusion–based method described by Seglen (Seglen 1976), with slight modifications, was used. Briefly, the rats were anesthetized with an intraperitoneal injection of ketamine (90mg/kg body weight) and xylazine (10mg/kg body weight) solution. The liver was perfused via the vena portae for 10min with a perfusion buffer I (0.14M sodium chloride, 6.7mM potassium chloride, and 10Mm HEPES), adjusted to pH 7.5 with 2.4M ethylene glycol tetraacetic acid (EGTA) at 39ºC. Subsequently, perfusion was continued with a collagenase buffer, consisting of 67mM sodium chloride, 6.7mM potassium chloride, 100mM HEPES, albumin (0.5%) adjusted to pH 7.6, and 4.8mM $CaCl2 \cdot 2H2O$, at 39ºC for 7min. The flow rate for the perfusion buffers was 10mL/min. After perfusion the liver was removed from the animal and dissociated in cold perfusion buffer I with 10g/L of albumin. The resultant cell suspension was filtered through gauze, centrifuged for 10min at 50g, washed once with medium, centrifuged again, and resuspended in medium in a final concentration of not more than $3.5x10^6$cells/mL. For the enrichment of the final hepatocyte population, an additional Percoll-step was included by layering 5mL of cell suspension over a 25% Percoll solution. After centrifuging at 1300 g at 4ºC for 20 min, hepatocytes were obtained as a pellet. The cell pellet was diluted in phosphate-buffered saline (PBS), centrifuged for 10 min at 50 g, and washed twice with PBS for removing the Percoll solution. Finally, the cells were harvested in supplemented Williams' E medium for culturing. The cellular viability of the isolated hepatocytes was assessed by trypan blue exclusion; routinely, values within an 85–95% range were obtained.

2.2. Cell culture

Freshly harvested hepatocytes were cultured with Williams' E medium supplemented with 10% fetal bovine serum (FBS) (v/v), 1.4µM hydrocortisone, 0.032U/mL insulin, 15mM HEPES, 1mM sodium pyruvate, 1mM of nonessential aminoacids (NEAA), and antibiotics (100U/mL penicillin/100mg/mL streptomycin and 40 mg/mL gentamicin) (Williams' E complete medium), or Vito 142 basal medium (Vito medium) (Biochrom AG, Berlin, Germany) with the corresponding supplement supplied by the manufacturer and antibiotics (100U/mL penicillin/100 mg/mL streptomycin and 40 mg/mL gentamicin), added accordingly. In static monolayer cultures (two-dimensional (2D) cultures), cells were seeded onto Matrigel®-precoated culture plates at a density of $5x10^4$cells/cm^2. Hepatocytes were left untreated for at least 12h at 37ºC in a humidified atmosphere with 5% CO_2 in air, allowing cell attachment. The medium was changed the following day to remove unattached cells. The culture medium was renewed every 24h, and the cells were routinely examined under phase contrast microscopy before every culture medium renewal. The supernatants and cells were collected according to the protocol at the indicated time points and stored at -20ºC for further assays. In stirred tank cultures (3D cultures), single-cell suspensions were seeded in 125mL spinner vessels or in a 250mL stirred tank bioreactor. An inoculum of $1.2x10^5$cells/mL or $2.4x10^5$cells/mL was used for a final volume of 70 or 200mL, in the spinner vessels or in the bioreactor, respectively, with supplemented medium at 15% FBS (to promote cell aggregation). Stirred tanks were placed on a magnetic stirrer, agitated at 60rpm, and kept at 37ºC in a humidified atmosphere of 5% CO_2 in air. After 3h in culture, to avoid settling of the cells, the stirring rate was increased to 80rpm. After 24h, 50% culture medium was changed and added to a final culture volume of 125 or 250mL, in the spinner vessels or in the bioreactor, respectively, and the FBS concentration was adjusted to 10% (v/v). To maintain the aggregates, the operational mode applied was a 50% medium substitution (refeed mode) every 4 days, for nutrient availability and to decrease the accumulation of bioproducts of cellular metabolism that can be toxic to the cells.

Cells were counted using a Neubauer counting chamber, and the cell viability was determined by the trypan blue exclusion method. Cell counting data was presented as percentage of cell survival considering cell number at day 1 as 100%.

2.3. Cultures in the bioreactor

To ensure a fully controlled cell culture environment, a glass vessel (**Fig. 2.1**) that could be adapted to a commercially available bioreactor control unit (B-DCU; B-Braun Biotech International, Melsungen, Germany) was designed and developed in our laboratory.

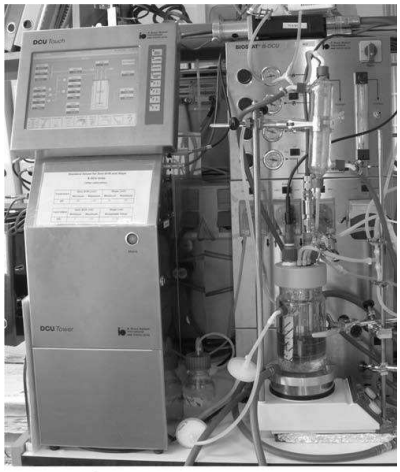

Figure 2.1.: Minibioreactor apparatus. (A) Bioreactor controller module used to control pH, pO2, and temperature. (B) Glass vessel with multiple (up to eight) upper cap ports for different applications (e.g., pH and pO2 meters and sampling tube) and a water jacket where temperature is controlled by a thermocirculator bath.

The internal geometry and the stirrer (paddle impeller) of the vessel are similar to the commercially available spinner vessels. The vessel has multiple (up to eight) upper cap ports for different applications, such as pH and pO_2 meters (Mettler-Toledo, Urdorf, Switzerland), that allow online measurement and control of these parameters, as well as easy sampling and the addition or removal of medium or other supplements or solutions. The pH is kept at 7.4 by injection of CO2 and addition of base solution (NaOH, 2M). The dissolved oxygen concentration is

maintained at 30% via surface aeration with air. The temperature was kept at 37ºC by water recirculation in the vessel jacket controlled by a thermocirculator bath. The bioreactor controller unit was used to monitor and control pH, pO_2, and temperature. Data acquisition and process control were performed using Multiple Fermenter Control System for Microsoft Windows (MFCS)/Win Supervisory Control and Data Acquisition (SCADA) software (B-Braun Biotech International).

2.4. Determination of lactate dehydrogenase activity

The release of intracellular enzymes – in particular, lactate dehydrogenase (LDH) – in the culture supernatant can be correlated with cell viability along the culture. This approach assumes that higher rates of release of enzymatic activity correspond to increased cellular damage and thus a loss in culture viability (Racher 1998). Hence, the extent of cell lysis was assessed by determination of LDH released to the medium as previously described by Vassault (Vassault 1983).

2.5. Determination of albumin secretion and urea synthesis

The secretion of albumin from hepatocytes was measured by an enzyme-linked immunosorbent assay (ELISA) using NEPHRAT albumin test kit (ref. NR002; Exocell, Philadelphia, PA). The assay was performed according to the manufacturer's description. The results were expressed as mg/day/10^6cells at the indicated time point. The urea synthesis rate was determined using a quantitative colorimetric urea kit (QuantiChrom™ Urea Assay Kit, DIUR-500, ref DIUR-500; BioAssay Systems), according to the manufacturer's instructions. The results were expressed as mg/day/10^6cells at the indicated time point.

2.6. Testosterone hydroxylation

Testosterone is regio- and stereoselectively metabolized by CYPs to several hydroxylated metabolites that were extracted and analyzed. In this study, 4-

androstene-3,17-dione (androstenodione), and 2α, 7α, 6β, and 16β-hydroxytestosterone were analyzed. Briefly, 250 µM testosterone dissolved in culture medium was added to cells and incubated for 2h at 37ºC. Hydroxylated metabolites were extracted with dichloromethane. After centrifugation (2000g, 5min), the organic phase was collected to a new clean tube and allowed to evaporate. The pellets were resolubilized in a mixture of methanol and water (50%/50%, v/v) for high-performance liquid chromatography (HPLC) analysis. Separations were performed on a 250x4mm RP18 Lichrocart, 5µm column using a Merck Hitachi LabChrom Elite chromatograph equipped with an autosampler, column oven, and diode array detector. Data analysis was performed with EZChrom Elite data system.

2.7. 7-ethoxycoumarin-O-deethylase (ECOD) activity

7-ethoxycoumarin-O-deethylase (ECOD) activity was measured according to Gomez-Lechon et al. (Gomez-Lechon *et al.* 1997) with slight modifications. Salicylamide (1.5mM) was added to the medium to prevent conjugation of 7-hydroxy metabolites (7-HC) of 7-ethoxycoumarin (Burke and Orrenius 1978). Therefore, hydrolyzation treatment with β-glucuronidase/arylsulfatase was overcome. The activity is expressed as nmol of 7-hydroxycoumarin formed per hour and per 10^6cells.

2.8. Uridine diphosphate glucuronoltransferase activity

The uridine diphosphate glucuronoltransferase (UGT) activity was determined by quantification of the substrate, 4-methylumbelliferone (4-MU), before and after cell incubation with the substrate. The procedure was performed according to Gomez-Lechon et al (Gomez-Lechon *et al.* 1997) with slight modifications. Briefly, a 100µM solution of 4-MU in 0.01M PBS was incubated with cells for an hour at 37ºC. Samples were measured by fluorescence with emission at 450nm and excitation at 320nm. The 4-MU remaining concentration was determined based on a standard

curve generated in PBS spiked with 0, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100µM. The activity is expressed as nmol of 4-MU metabolized per hour and per 10^6cells.

2.9. Preparation of test compounds

All compounds used for metabolic stability assays were purchased from Sigma-Aldrich (Taufenkirchen, Germany). Stock solutions were prepared by dissolving diphenhydramine in acetonitrile (Carl Roth, Karlsruhe, Germany) and troglitazone in dimethyl sulfoxide (Carl Roth) at a final concentration of 10mM. The highest solvent concentration used was 0.05% (v/v).

2.10. Metabolic stability assay

Stock solutions (10mM) in acetonitrile and dimethyl sulfoxide were diluted in Williams' E complete medium to obtain working solutions. The concentration of final compounds during each assay was 5µM.

In 2D cultures (24-well plates), the reaction was initiated by the addition of 250µL of test item working solution at a concentration of 5µM to the pre-incubated hepatocytes at 37ºC for 5min. For 3D cultures, 125µL of the compounds stock solution (10mM) was added to the 250mL bioreactor 3D culture resulting in a final concentration of 5µM. Cell concentration in each culture system was as described in the Cell Culture section. Controls consisting of compounds dissolved in supplemented medium, but not in the presence of cells, incubated at 37ºC, were always performed in parallel. To stop the reaction, 250µL of the test item samples was removed from the incubations at the respective time points and processed for acetonitrile precipitation. Isolation of the test items was performed by addition of 107µL acetonitrile containing the internal standard griseofulvin (ISTD, 1µM) to 250µL sample and calibration standard, respectively. After vigorously shaking (10s), sonification (10s), and centrifugation (5000 g, 10 min), an aliquot of the particle-free supernatant was subsequently subjected to liquid chromatography-mass

spectrometry (LC-MS/MS) for analysis. The HPLC system consisted of a MS Plus pump (Surveyor, Waltham, MA) and an auto sampler (Surveyor). Mass spectrometry was performed on a TSQ Quantum Discovery Max triple quadruple mass spectrometer equipped with an electrospray (ESI) interface (Thermo-Finnigan, Waltham, MA) and connected to a PC running the standard software Xcalibur 1.4.

2.11. Statistics

The data presented for the different culture parameters represent mean values for three independent experiments with two determinations each. Comparison of mean values between 2D monolayer, 3D spinner, and 3D bioreactor cultures, as well as the effect of inoculum concentration study, was performed using an appropriate one-way analysis of variance (ANOVA) test. Data evaluation between 2D and 3D spinner cultures maintained with Williams' E and Vito medium, on the other hand, was performed using a two-way ANOVA test. Multiple comparisons between groups in each case were performed using a Tukey's honestly significant difference test. Level of confidence was set at α=0.05 for all tests, with p-values lower than 0.05 considered statistically significant.

3. RESULTS

3.1. 3D culture optimization

Evaluation of culture conditions that better granted specific hepatocyte cellular functions, using previously established biochemical assays, was examined. Albumin secretion and urea secretion by hepatocytes are indicators for long term functional performance of hepatic cultures, whereas the activity of phase I (such as CYP 450) and phase II enzymes mirrors the capacity of hepatocytes to metabolize xenobiotics.

On the other hand, general culture parameters, such as LDH, glucose, and lactate levels, have also been considered. The spheroid performance as 3D

structures cultured in spinner vessels (3D cultures) was evaluated against conventional cultures in monolayer static conditions (2D cultures).

Immediately after isolation, cells were inoculated in 3D and 2D conditions and allowed to stabilize overnight. Cultures were maintained for 2 weeks.

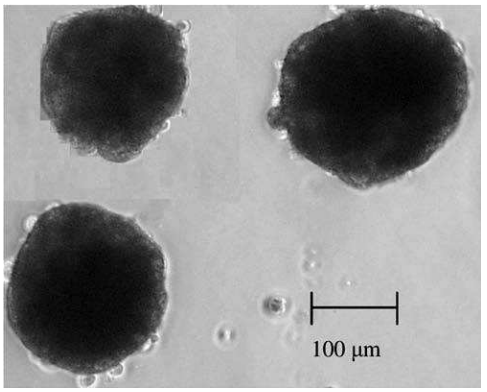

Figure 2.2.: Phase contrast microscopy with an amplification of 100x, of a 3D hepatocyte spheroid, cultured in stirred tanks and sample collected at day 3 of culture.

Stirring rate in suspension cultures was kept at 60 rpm overnight. On day 1, cell adhesion to the plastic surface of the culture dish was observed in static conditions, whereas in suspension cultures small tightly packed groups of cells, of approximately 100μm, were detected. During the remaining culture time, stirring rate was increased to 80 rpm in 3D cultures; spheroids became larger, reaching sizes of 100–300μm in diameter (**Fig. 2.2**). Such size range was not critical regarding the formation of necrotic centres as assessed by LDH (data not shown).

In both monolayer (2D) and spheroid (3D) cultures, no cell proliferation was observed, as expected for hepatocyte primary cultures. However, in general, approximately 2.5-fold higher viability for a longer period of time (as shown in **Fig. 2.5 D**) could be achieved when hepatocytes were cultured as 3D structures.

3.1.1. Effect of inoculum concentration, impeller type and medium composition on cultures performance.

Two different inoculum concentrations ($1.2x10^5$ and $2.4x10^5$ cells/mL) and two impeller types (ball and paddle) were compared when optimizing the 3D culture conditions. As for culture performance, the albumin secretion levels and ECOD activity did not vary much with different impeller (data not shown) or inoculum concentrations within 3D cultures, although they were consistently higher in 3D than in 2D conditions (**Fig. 2.3**) ($p<0.05$).

Albumin production decreased dramatically in 2D cultures, reaching residual levels after 2–3 days in culture, whereas 3D cultures allowed albumin secretion to continue for more than 8 days in culture (**Fig. 2.3 A**). ECOD activity, on the other hand, was generally five times higher in 3D cultures than in 2D cultures (**Fig. 2.3 B**) ($p<0.05$). No significant difference could be observed between cultures with an inoculum of $1.2x10^5$ and $2.4x10^5$ cells/mL at days 2 and 7 of culture ($p>0.05$). Urea synthesis was significantly higher in spheroid cultures with an inoculum of $1.2x10^5$ cells/mL ($p<0.05$). Moreover, overall synthesis of testosterone metabolites was also higher in the later culture conditions, detectable up to day 12.

In summary, spheroid cultures, with an inoculum of $1.2x10^5$ cells/mL using a paddle impeller, resulted in enhanced hepatocyte functionality.

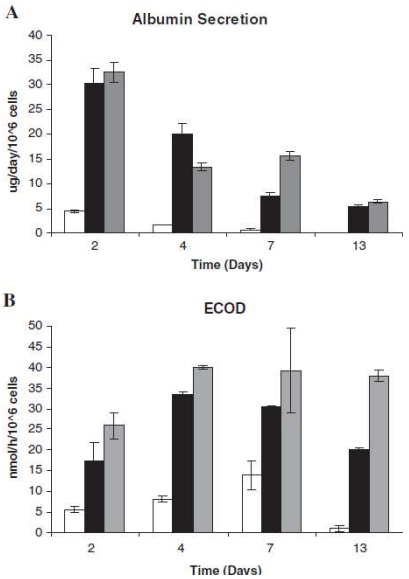

Figure 2.3.: Effect of culture time in (A) albumin secretion and (B) activity of phase I enzyme ECOD for cells cultured in monolayer 2D(white bars) and spinner vessels 3D with paddle impeller with an inoculum concentration of $2.4x10^5$ cells/mL (black bars) and $1.2x10^5$ cells/mL (gray bars).

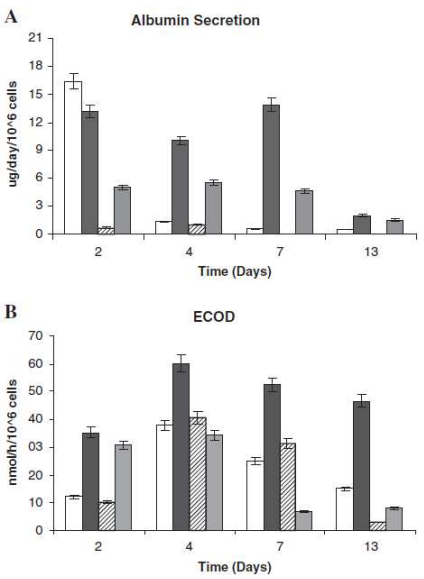

Figure 2.4.: Effect of culture time on (A) albumin secretion and (B) activity of phase I enzyme ECOD for cells cultured in Williams' E medium in monolayer 2D (white bars) and spinner vessels 3D (black bars), Vito medium in monolayer 2D (hatched bars), and spinner vessels 3D (gray bars).

Regarding medium composition, Williams' E medium and Vito medium were selected. Our results showed that although spheroids cultured in Vito medium allowed 20% higher survival rate maintenance, 3D cultures in Williams' E resulted in significantly higher albumin secretion (**Fig. 2.4 A**) ($p<0.05$), for longer periods of time, never reaching residual levels before day 9, whereas with Vito medium residual levels were observed after 3 days in culture.

Overall, CYP 450 activity, determined by measuring ECOD (**Fig. 2.4 B**) and testosterone metabolites (data not shown), was significantly higher in 3D when Williams' E medium was used ($p<0.05$). Therefore, Williams' E medium was adopted in the following cultures. However, in cell cultures a compromise between cell viability and cell functionality must be achieved; in hepatocyte cultures for pharmacological purposes, it is critical to keep hepatocytes functionality for as long

as possible; therefore, Williams' E medium overall results showed greater advantage over Vito medium results.

3.2. Metabolic performance of bioreactor cultures

Because the optimized 3D cultures in spinner vessel improved liver-specific functions, we went forward to apply the optimized parameters to a bioreactor (fully controlled environment) (**Fig. 2.5 A–C**) where an equivalent or better culture performance could be expected. Cultures were maintained for a total of 21 days in the bioreactor, as well as in spinner vessels and in monolayer, herein used as control cultures. Higher survival rates could be achieved when hepatocytes were cultured as 3D structures (**Fig. 2.5 D**), confirming the results obtained in spinner vessels described above.

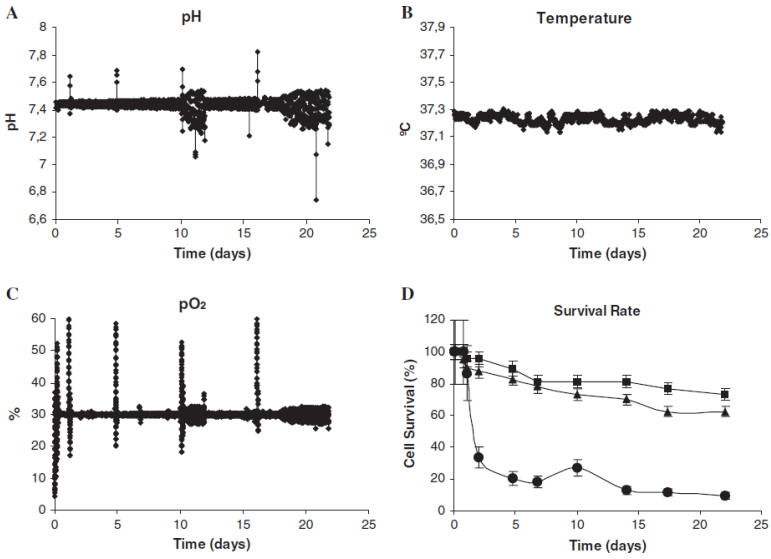

Figure 2.5.: Profiles of (A) pH, (B) jacket temperature, and (C) dissolved oxygen (pO2) collected in the bioreactor used for the cultivation of hepatocyte spheroids. (D) Survival rate curves of rat hepatocytes cultured in monolayer 2D (•), bioreactor 3D (■), and spinner vessels 3D (▲) culture systems.

In both 3D cultures, after an adaptation phase that lasted about 3–4 days, cells showed relatively stable metabolic activity over at least 10 more days of culture. LDH leakage rates were high at day 1 but declined during the culture time, followed by a slow increase from day 14 in 3D cultures.

Biochemical data for days 4–14 showed no significant difference among 3D cultures, whereas 2D cultures reached residual levels after 2–3 days in culture without recovering. In 3D bioreactor cultures, urea secretion (**Fig. 2.6 A**) values were maintained at a stable level, showing only a slight decrease from day 14 onward. Still, urea production was significantly higher in 3D bioreactor than in the other two culture systems ($p<0.05$). Albumin production (**Fig. 2.6 B**) in 3D bioreactor culture after day 1 was maintained at nearly constant levels throughout culture time, decreasing approximately 75% only on day 21. Significantly higher albumin production values were obtained in 3D culture systems ($p<0.05$), although even higher in the 3D bioreactor cultures ($p<0.05$).

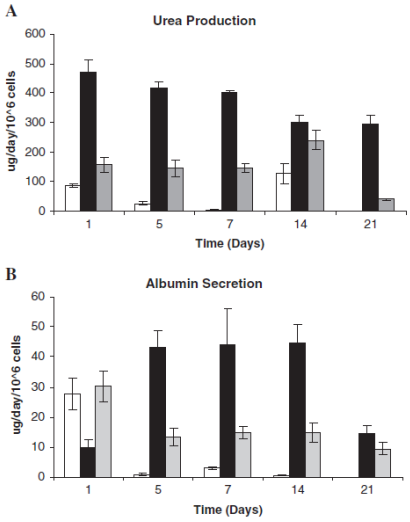

Figure 2.6.: Functional capacity of hepatocytes over long-term cultivation periods was assessed by determining (A) urea production and (B) albumin secretion by the cells cultured in monolayer 2D (white bars), bioreactor 3D (black bars), and spinner vessels 3D (gray bars) culture systems.

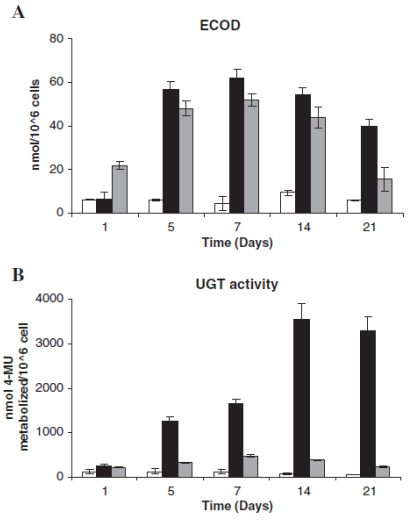

Figure 2.7.: Hepatic metabolic function was assessed by determining the activities of (A) phase I enzymes ECOD and (B) phase II enzymes UGT in cells cultured in monolayer 2D (white bars), bioreactor 3D (black bars), and spinner 3D (gray bars) culture systems.

The testosterone metabolism and ECOD activity are critical routine assays to determine the activity of the CYP 450 (CYP) isozyme system. In **Figure 2.7 A**, it is shown that ECOD activity was significantly improved in 3D cultures when compared

to 2D cultures from day 1 onward ($p<0.05$), with even higher values being obtained in the bioreactor ($p<0.05$).

Concerning the testosterone metabolism, metabolites such as androstenodione and 2α, 7α, 6β, and 16β-hydroxytestosterone were considered. The formation of those metabolites by isolated hepatocytes cultured in 2D and 3D conditions (spheroids cultured in spinners vessels or in the bioreactor) during 21 days was quantified. The quantity of hydroxytestosterone metabolites produced by freshly isolated cells was variable depending on the metabolite and on the culture system. However, clearly testosterone hydroxylation profiles were always higher in both 3D systems. In monolayer cultures, results showed that the testosterone metabolism dropped dramatically after day 4 in culture, where no more activity could be detected. In contrast, in 3D bioreactor cultures, activity increased with culture time to approximately 4.5-fold that of fresh cells after 3–4 days in culture. Testosterone metabolites were detected up to day 16 in the bioreactor culture and up to day 12 in the spinner vessels. Metabolism of testosterone to 6β and 2α-hydroxytestosterone was similar in both 3D culture systems, showing average values of 4.4pmol/min/10^6cells and 4.6pmol/min/10^6cells, respectively. On the other hand, metabolism of testosterone by combined CYP 2B1, 2C11, and 2B2 activity (resulting in the formation of androstenodione) in the bioreactor was 5.2-fold greater than in the spinner vessel. Metabolism of 7α-hydroxytestosterone and 16β-hydroxytestosterone in the bioreactor was 3.2- and 2.7-fold greater than in the spinner vessel, respectively.

Among phase II enzymes, UGT is involved in conjugation processes of substrates. **Figure 2.7 B** shows results for UGT activity. A significant improvement of UGT activity in the bioreactor system could be observed ($p<0.05$) reaching a maximum activity at day 14 when compared to the other two culture systems. Although a considerably lower activity was found, the hepatocytes cultured on 3D structures in the spinner vessel still exhibited a UGT activity significantly higher than those cultured in monolayer ($p<0.05$).

3.3. Metabolic stability assays

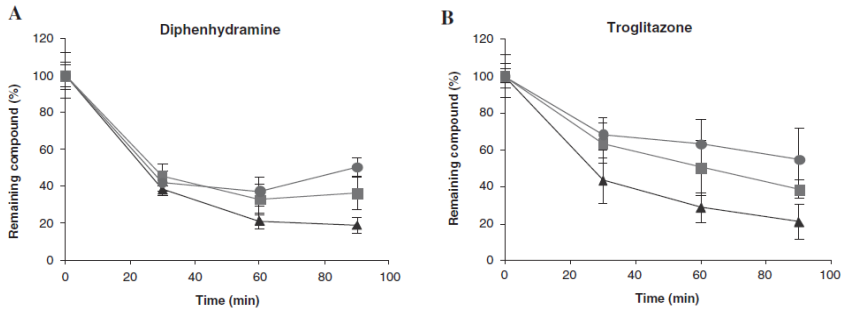

Figure 2.8.: Metabolic stability of (A) diphenhydramine and (B) troglitazone observed in hepatocyte primary cultures in bioreactor 3D at day 2 (•) and day 21 (▲) of culture; and in monolayer 2D at day 2 (■) of culture.

To further validate the newly developed bioreactor-based 3D primary hepatocytes culture system, metabolic stability of a couple of compounds (troglitazone and diphenhydramine) was examined both in the 3D bioreactor and in the 2D culture system. The incubations were always performed in supplemented medium because this was mandatory for the bioreactor and data had to be comparable. No influence of the incubation medium in the compounds metabolism was observed. In **Figure 2.8 A** and **B**, it is shown that, at day 2 of culture, cells in both culture types were able to metabolize the compounds equally. Moreover, at day 21 bioreactor cells in 3D cultures were still able to metabolize the compounds, whereas in 2D cultures, because cell viability was very low (**Fig. 2.5 D**), the same could no longer be performed.

4. DISCUSSION

An important scientific achievement in this study was the development of an alternative *in vitro* hepatocyte culture system that can be used for both toxicological and pharmacological studies.

The choice of a 3D system was based on the fact that hepatocytes retain both high viability and differentiated liver functions when cultured as 3D aggregates. This was shown by Li et al. (Li *et al.* 1992) who successfully cultured viable spheroids on non-tissue culture plastic plates for 1 month. Li et al. and Yoffe et al. (Yoffe *et al.* 1999) were also among the first authors to report hepatocyte aggregates cultured in rotating wall vessels. Aggregates could be cultured for a prolonged period with differentiated cell morphology and functions. The advantage of our system over previously described *in vitro* models is that it conjugates several characteristics that are important for functional hepatocytes culture and for culture practicability in general. The cells were cultured in a fully controlled environment bioreactor where sampling throughout the whole culture time was made possible. It is a setup based on commercially available equipment and therefore accessible to any laboratory or company. Such system decreases the biological cell-to-cell and culture-to-culture variability that has thus far limited the size of cell based experiments. Further, not only this minibioreactor did allow for oxygen control in suspended cultures (Moreira *et al.* 1995; Balis *et al.* 1999) but also the stirred tank environment enabled a better medium oxygenation further resembling the physiological blood flow (Bader *et al.* 1999). It has been shown that the absence of oxygen in culture can lead to a cellular metabolic state of sensitive or even rapid hypoxic injury, due to the depletion of dissolved oxygen; regular culture conditions do not allow this control as a bioreactor would (Cho *et al.* 2007).

In this study, before the application of the 3D hepatocyte cultures to the bioreactor system, some culture parameters were tested in spinner vessels to establish a culture system for hepatocytes *in vitro* maintenance. The maintenance of a viable cell culture in suspension conditions requires optimization of several

cultivation parameters. In particular, for spheroid cultures, it is mandatory to control their size hydrodynamically, to avoid necrotic centers resulting from oxygen and nutrient transport limitations. Experimental parameters such as stirring rate and impeller design can be optimized to circumvent such drawbacks without the decrease in culture viability (Moreira *et al.* 1995).

Moreover, medium composition and inoculum concentration are also crucial parameters to be considered when developing a new culture system. Therefore, parameters such as inoculum concentration, medium formulation, impeller type, and stirring rate were studied herein.

Concerning the media, higher survival rates, but lower hepatocyte-specific activities were observed with Vito medium than with Williams' E medium. Probably, it was due to the fact that Vito medium was designed for culturing human rather than rat hepatocytes, although it was specially developed for culturing hepatocytes in bioreactors (Zeilinger *et al.* 2002). On the other hand, both the lower inoculum concentration and paddle impeller type showed better results (**Fig. 2.3**), the lower inoculum being an advantage to minimize the low availability of hepatocytes.

In this context, the optimized conditions ($1.2x10^5$ cells/mL inoculum, cultured in supplemented Williams' E medium with a paddle impeller) were used in the bioreactor.

Hepatocyte-specific functions such as albumin and urea secretion are important indicators when evaluating hepatocyte cultures. Albumin secretion is a general marker of protein synthesis in hepatocytes as it is synthesized almost entirely by the liver. It is a source of amino acids for various tissues. Decreased albumin level can serve as an index of impaired synthetic protein capacity by the liver (Weigand and Otto 1974). Likewise, the conversion of ammonia to urea is a vital liver function. The ammonia produced through amino acid deamination can be detoxified by the combination of ammonia with CO_2 to form urea in the liver (Watts *et al.* 1995). In this study, albumin secretion rapidly decreased in monolayer cultures but not in 3D cultures. This is not an unexpected finding because studies of the albumin metabolism have shown that the synthesis and albumin secretion by isolated

hepatocytes occur only in early culture phases (Weigand and Otto 1974). However, in our culture system this effect was circumvented because albumin secretion could be detected throughout a significant period of time, decreasing only after day 14.

Although higher values were observed in both 3D culture systems, in contrast to the 2D system, the bioreactor clearly showed higher values than the spinner vessels. Accordingly, in both 3D cultures urea production levels were clearly higher than those produced by hepatocytes cultured in monolayer. Still, higher levels were observed in the bioreactor than in the spinner vessel, suggesting that not only does growth in 3D structures enhance liver-specific functions, but also a fully controlled environment, resembling the *in vivo* conditions, is an issue in this type of cultures. One parameter further controlled in the bioreactor is the dissolved oxygen, a key factor on hepatocyte cell long-term cultures (Bader *et al.* 1999). Therefore, an oxygenated environment may be the reason why better results are observed in the bioreactor.

Biotransformation of xenobiotics by cultured hepatocytes can be assessed in terms of activity using specific substrates. Synthetic substrates metabolism by primary cultures of hepatocytes cultured in plates and in stirred tanks (spinner vessels and bioreactor) was examined by measuring phase I and phase II enzyme activities. The functionality of CYP 450 enzymes in the culture systems was assayed via the regiospecific hydroxylation of testosterone and the 7-ethoxycoumarin O-deethylation (ECOD activity), whereas phase II enzymes were assayed by UGT. Metabolism of several endo- and exogenous substances is carried out by a set of phase I and phase II enzymes. Their activities determine the overall therapeutic and toxic profiles of a drug (Schenkman 1993). The 6β-hydroxylation of testosterone is predominantly mediated by CYP 3A, the major isoform being 3A4 in humans (Waxman *et al.* 1991) and 3A1 in rats (Sonderfan *et al.* 1987). Oxidation of testosterone to 4-androstene-3,17-dione in rats is predominantly catalyzed by CYP 2B1 with minor contributions from CYP 2C11 and CYP 2B2 (Sonderfan *et al.* 1989). In human hepatocytes, CYP 2C19 and to a lesser extent CYP 2C9, are responsible for production of 4-androstene-3,17-dione, with potential contribution from CYP 2B6

(Yamazaki and Shimada 1997). 2α-Hydroxylation is predominantly mediated by CYP 2C11 in rat (Sonderfan *et al.* 1989). A CYP 2C11 orthologous enzyme has not yet been identified in humans, but 2α-hydroxylation is presumably mediated by an enzyme belonging to the 2C subfamily (Raucy *et al.* 2002; Thomas *et al.* 2005). ECOD activity in rat hepatocytes is mostly mediated by CYP 1A and 2A families (Rogiers *et al.* 1986). Research in the area of drugs biotransformation in static 2D primary human and rat hepatic sandwich cultures has revealed a preservation of the major forms of phase I and phase II enzymes that respond to rifampicin by increasing their activities (Kern *et al.* 1997). However, the long-term maintenance of the phase I enzymes Ethoxyresorufin-O-deethylase (EROD) (CYP1A1/2) and ECOD (CYP2B1/2), as well as of the phase II enzyme UGT, was achieved in sandwiched primary porcine hepatocyte cultures in a dynamic flat-sheet bioreactor with a gas-permeable membrane (Langsch and Bader 2001) and in a small-scale bioreactor with an hepatic sandwich model and a gaspermeable membrane (Schmitmeier *et al.* 2006). Similarly, our work using a fully controlled bioreactor showed that these activities were also well preserved in rat hepatocytes. Here, the importance of an adequate oxygenation for the phase I, but mostly phase II metabolism, is emphasized by the lower activities in spinner vessels and by the lack of an effective phase I and phase II enzymatic activities in the conventional 2D monolayer culture dishes after 2–3 days in culture.

The metabolic stability of a drug candidate is an important consideration in determining its potential for human use. Those studies are usually conducted during earlier stages of drug development to allow the selection of structures with the most appropriate stability for further development.

Because of the presence of all hepatic drug metabolizing enzymes and cofactors at physiological levels, intact hepatocytes represent a more relevant experimental system than liver microsomes for general metabolic stability screening. An interesting achievement in the application of intact hepatocytes for the evaluation of metabolic stability is the incubation of the test compound and hepatocytes in 100% human serum, therefore providing an experimental condition similar to humans *in vivo*. For

instance, it has been reported that hepatic clearance *in vivo* can be predicted more accurately from data obtained with hepatocytes incubated in serum than from data obtained in the absence of serum, using either rat or human hepatocytes (Li 2007). Thus, the use of a compound whose behavior in metabolic stability assays is known can be considered as reference for the evaluation of a culture system under development. In this work, metabolic stability of reference compounds (diphenhydramine and troglitazone) was evaluated with the new *in vitro* culture system using serum-supplemented medium. In fact, the results clearly showed that compound clearance was as *in vivo*, thus highlighting its potential for pharmacological studies.

This work shows that it is possible to culture primary rat hepatocytes as 3D structures using a fully controlled bioreactor and that this culture strategy improves hepatocyte functionality. By more closely resembling the *in vivo* environment, due to its three-dimensionality and cell–cell interaction, this model supported long-term viability and the maintenance of several differentiated hepatocytes properties, including xenobiotic metabolism, for up to 21 days. In contrast, traditional monolayer cultures lost these properties after 3-4 days in culture. Therefore, this system offers a much improved alternative culture system for predictive *in vitro* studies, to study mechanisms of CYP isoforms and to perform metabolic stability studies. It is also a new method for testing drug interaction under 3D conditions, because it has the advantage of offering a better *in vitro–in vivo* correlation. This culture system can be implemented for primary human hepatocytes avoiding interspecies differences. Another promising application is for human stem cell–derived hepatocytes culture, which is currently being assessed in our laboratory.

5. ACKNOWLEDGMENTS

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III

RAT HEPATOCYTES IN 3D CO-CULTURES

This chapter is based on the following manuscript:

Merging bioreactor technology with 3D hepatocyte-fibroblasts culturing approaches: enhanced in vitro models for Toxicological Applications

Leite SB, Teixeira AM, Miranda JP, Tostoes RM, Clemente JJ, Sousa MS, Carrondo MJT, Alves PM. (2011) Toxicology in Vitro 25(4):825-32.

Sofia B. Leite designed, performed and analysed the results of this study. The remaining authors of this work had collaborated in different stages of the study

ABSTRACT

During the last years an increasing number of *in vitro* models have been developed for drug screening and toxicity testing. Primary cultures of hepatocytes are, by far, the model of choice for those high-throughput studies but their spontaneous dedifferentiation after some time in culture hinders long-term studies. Thus, novel cell culture systems allowing extended hepatocyte maintenance and more predictive long term *in vitro* studies are required.

It has been shown that hepatocytes functionality can be improved and extended in time when cultured as 3D-cell aggregates in environmental controlled stirred bioreactors. In this work, aiming at further improving hepatocytes functionality in such 3D cellular structures, co-cultures with fibroblasts were performed. An inoculum concentration of $1.2x10^5$ cell/mL and a 1:2 hepatocyte:mouse embryonic fibroblast ratio allowed to improve significantly the albumin secretion rate and ECOD (phase I) and UGT (phase II) enzymatic activities in 3D co-cultures, as compared to the routinely used 2D hepatocyte monocultures. Significant improvements were also observed in relation to 3D monocultures of hepatocytes. Furthermore, hepatocytes were able to respond to the addition of Beta-Naphtoflavone by increasing ECOD activity showing CYP1A inducibility. The dependence of CYP activity on oxygen concentration was also observed. In summary, the improved hepatocyte specific functions during long term incubation of 3D co-cultures of hepatocytes with fibroblasts indicate that this system is a promising *in vitro* model for long term toxicological studies.

CONTENTS

1. INTRODUCTION

Primary cultures of freshly isolated hepatocytes are currently the *in vitro* model of choice for drug screening applications. When cultured under well-defined and stringent conditions, these primary cells perform alike common hepatocyte cell lines (e.g. HepG2) in terms of ability to induce P450 (CYP) activity upon addition of specific compounds; however, they have improved synthesis and secretion rates of both albumin and urea as well as improved capacity for phase I metabolism (Donato *et al.* 1994; Viollon-Abadie *et al.* 2000; Gebhardt *et al.* 2003).

As explain on Chapter I, hepatocyte cultures of human origin can be obtained from different sources. However, these cells are rather difficult to obtain and often their profile is highly dependent on the donor sources presenting a high batch-to-batch variability. Primary cultures of rat hepatocytes are a good alternative often used by the pharmaceutical industry because (i) they have higher metabolic responses than common human cell lines and (ii) the inter-donor variability can be minimized by selecting animals of the same sex, age and similar feeding regimes (Coecke *et al.* 2000; O'Brien *et al.* 2004). Moreover, the strategies developed to improve hepatocytes functionalities can often be applied easily to primary human hepatocytes.

Conventional 2D *in vitro* models of hepatocytes present the major drawback of quick loss of expression of specific liver-functions (O'Brien *et al.* 2004). Strategies to decrease or, ideally, overcome, *in vitro* dedifferentiation of hepatocytes constitute a major goal in drug development. Several reports on the effect of (i) extracellular matrices (ECM) that promote cell adhesion (Skett 1994) and/or of (ii) 3D culture methodologies (cell spheroids) that allow for a closer to *in vivo* cell-cell contact (Rotem *et al.* 1992; Fischbach *et al.* 2007; Pampaloni *et al.* 2007; Yamada and Cukierman 2007; Lin and Chang 2008), in hepatocytes functionality, are available in the literature. In particular, different studies have shown that cell-cell interactions, especially heterotypic cell interactions (Bhatia *et al.* 1998), are more effective in the maintenance of hepatocyte functions than ECM configuration (Michalopoulos *et al.* 1979; Khetani and Bhatia 2008). Significant enhancements in hepatocytes

phenotype maintenance and function were described for co-cultures of hepatocytes with connective tissue or non-parenchymal cells (Bhatia *et al.* 1998; Bhandari *et al.* 2001; Lu *et al.* 2005; Khetani and Bhatia 2008). These cells are able to either secrete soluble signaling molecules (e.g. cytokines, hormones and growth factors) or provide other cell-associated signals (i.e., insoluble extracellular matrix or membrane bound proteins). Furthermore, the combined strategy of co-cultures and 3D cellular organization permits cell-to-cell interactions between both cell types at various points of contact, as oppose to the single anchorage site between co-cultured cells maintained in 2D (Lu *et al.* 2005).

Fibroblasts are the most common cell type of connective tissue with a crucial role in the synthesis of extracellular matrix for tissue remodeling and repair. Additionally, they are the main producers of hepatocyte growth factor (HGF) (Stoker *et al.* 1987), a molecule involved in anti-apoptotic mechanisms in hepatocytes (Hiramatsu *et al.* 2005), and as they have no ability of xenobiotic biotransformation their metabolism does not interfere in toxicological assays (Donato *et al.* 1990). Mouse fibroblasts have been successfully used as feeder cells of rat hepatocytes (Bhatia *et al.* 1998; Kang *et al.* 2004; Lu *et al.* 2005; Cho *et al.* 2008; Kidambi *et al.* 2009; Miyamoto *et al.* 2009). Also, the presence of human markers on feeder cells has been shown to improve rat hepatocyte functionalities (Michalopoulos *et al.* 1979). Recently, Cho et al. (Cho *et al.* 2010) reported uniform intracellular albumin staining and E-cadherin expression, increased liver-specific functions and active glycogen synthesis in the hepatocytes when they were co-cultured in 2D with fibroblasts in a controlled microenvironment and the heterotypic interface between both cell types was increased by the layered patterning technique.

Several bioreactors have been developed for culturing hepatocytes, in particular for cell therapy applications (e.g bioartificial livers) (De Bartolo *et al.* 2000; Gerlach *et al.* 2003; Son *et al.* 2006; Fiegel *et al.* 2008) or for pharmacological and toxicology studies (Brown *et al.* 2003; Schmitmeier *et al.* 2006). The bioreactor technology presents several advantages for cell culturing as it allows a well defined

environment with control of critical culture parameters such as oxygen concentration, pH and temperature (Sa Santos *et al.* 2005; Miranda *et al.* 2009; Miranda *et al.* 2010). In particular, it is known that an adequate oxygen delivery to hepatocytes in culture is imperative for the maintenance of liver-specific functions such as alanine transferase, aspartate aminotransferase or phase I and phase II biotransformation enzymes, as gene expression rapidly declines when the cells are exposed to low oxygen concentrations (Cho *et al.* 2007). Moreover, cultures performed in stirred tank bioreactors can be sampled over culture time, with minimal perturbation and without compromising the sterility of the culture. Additionally, these bioreactors can be instrumented with specific devices to support perfusion operation which has been shown to bring additional advantages to hepatocyte cultures (Tostoes *et al.* 2011).

Previous work from our group showed improved and extended functionality for hepatocytes cultured as 3D spheroids (or aggregates) in small scale stirred tank bioreactors (Miranda *et al.* 2009). In the present work we aimed at improving even further this *in vitro* model by combining it with a co-culture approach. Taking the advantages of an accurate system for oxygen monitoring and control, the effect of oxygen concentration in hepatocytes functionality was also assessed.

2. MATERIALS AND METHODS

2.1. Animals, Materials and Biologics

Wistar male rats (6-9 weeks old, with 200–300 g body weight) were obtained from the animal house of *Instituto de Higiene e Medicina Tropical* (Lisbon, Portugal). All animal experiments were performed according to experimental procedures approved by the Portuguese Ethics Committee on animal experimental research. Williams'E medium, Essential Medium Eagle (MEM), foetal bovine serum (FBS), phosphate-buffered saline (PBS) solution and trypsin-EDTA as well as the remaining medium supplements were purchased from Invitrogen (Glasgow, UK). Culture flasks were obtained from Nunc (Roskilde, Denmark). The cell membrane labeling kit (PKH67 Fluorescent Cell Linker Mini Kit) was purchased from Sigma.

2.2. CELL CULTURES

2.2.1. Rat hepatocyte isolation and 2D cultures maintenance

Hepatocytes were isolated from rats using the two-step collagenase perfusion–based method described by Seglen (Seglen 1976) with slight modifications. Briefly, the rats were anesthetized with an intraperitoneal injection of ketamine (90 mg/kg body weight) and xylazine (10 mg/kg body weight) solutions. The liver was perfused via the vena portae for 10 min with perfusion buffer I (0.14 M sodium chloride, 6.7 mM potassium chloride and 10 mM HEPES), adjusted to pH 7.5 with 2.4 mM EGTA at 37ºC. Subsequently, perfusion was continued with a collagenase buffer, consisting of 200 mg/L collagenase P (Roche applied Science), 67 mM sodium chloride, 6.7 mM potassium chloride, 100 mM HEPES, Bovine Serum Albumin (BSA, 200 g/L) adjusted to pH 7.6, and 4.8 mM calcium chloride dihydrated, at 37ºC for 7 min. The flow rate for the perfusion buffers was 10 mL/min. After perfusion the liver was removed from the animal and dissociated in cold perfusion buffer I with 10 g/L of albumin. The resultant cell suspension was filtered through gauze, centrifuged for 10 min at 50 g, washed once with medium, centrifuged again, and resuspended in medium in a final concentration of $3.5x10^6$ cells/mL. An additional Percoll-step was included for hepatocytes purification thereby eliminating both non-viable and non-parenchymal cells, by layering 5 mL of cell suspension over a 25% Percoll solution. After centrifuging at 1300 g at 4ºC for 20 min, the pellet of hepatocytes was diluted in PBS, centrifuged for 10 min at 50 g, and washed twice with PBS for removing the Percoll solution. Finally, the cells were resuspended in complete Williams' E medium (described below). The viability of the freshly isolated hepatocytes was assessed by counting the cells in a haemaocytometer using the trypan blue exclusion method; values within an 85–95% range were routinely obtained.

For 2D cultures, freshly harvested hepatocytes were seeded onto matrigel® pre-coated culture plates at a density of $5x10^4$ cells/cm^2 total cells. Hepatocytes were cultured with WilliamsE medium supplemented with 10% FBS (v/v), 1.4 µM

hydrocortisone, 0.032 U/mL insulin, 15mM HEPES, 1mM sodium pyruvate, 1mM non-essential aminoacids, and antibiotics - 100 U/mL penicillin, 100 mg/mL streptomycin and 40 ug/mL gentamicin (WE complete medium). In 2D cultures, hepatocytes expressed liver-specific functions during approx. 7 days.

2.2.2. Fibroblasts cultures

Three types of fibroblasts were tested: mouse embryonic fibroblasts (mef), human foreskin fibroblasts (hff) and NIH 3T3 fibroblasts. Mouse embryonic fibroblasts were isolated after fibrous tissue trypsin digestion of fetuses with 12 to 14 gestation days using the methods described by Robertson (Robertson 1987). Both hff and NIH 3T3 fibroblasts were purchased from ATCC.

Fibroblasts were routinely cultured in tissue culture flasks and maintained in medium MEM supplemented with 10% (v/v) FBS and 100 U/ml (Penicillin/Streptomycin). The culture medium was exchanged every 3 days and fibroblasts were diluted 1:2 (first passage) and 1:3 upon (first passage) confluence. For co-cultures, confluent fibroblasts were growth arrested by adding 100 µM of Mytomicin for 3 hours into the culture medium. Afterwards, cells were rinsed three times in PBS, trypsinized, gently mixed with the hepatocytes suspension and inoculated in spinner vessels or bioreactors as described below.

2.2.3. 2D Co-cultures of Hepatocytes and Fibroblast

Freshly harvested hepatocytes and fibroblasts were seeded simultaneously onto matrigel® pre-coated culture plates at a density of $1.25x10^5$ cells/cm^2, according to Bhatia (Bhatia *et al.* 1998). After seeding, cultures were kept untouched for at least 12h at 37°C in a humidified atmosphere with 95% air and 5% CO_2, to allow cell attachment. Unattached cells were then removed by medium exchange. The culture medium was renewed every 24h and cells were routinely examined under phase contrast microscopy before every culture medium renewal. Culture supernatant and cells were collected according to the protocol at the same time points as 3D cultures

and stored at -20°C for further assay. In 2D co-cultures, hepatocytes expressed liver-specific functions during approx. 10 days.

2.2.4. 3D Co-cultures of Hepatocytes and Fibroblast

3D co-cultures of hepatocytes/fibroblasts were performed in bioreactor and spinner vessels. Spinner cultures were maintained inside an incubator at 37ºC with a humidified atmosphere of 5% CO_2 in air. The inoculation of both spinner and bioreactor vessels was performed as follows: single-cell suspensions of hepatocytes and fibroblasts were mixed together at an agitation rate of 60 rpm in 70/200 ml (spinner/bioreactor) of Williams E complete medium supplemented with 15% (v/v) FBS to promote cell aggregation. Three hours post inoculation, the agitation rate was increased to 80 rpm to avoid sedimentation of the cell spheroids and after 24 h, half of the culture medium was replaced by fresh medium (supplemented with FBS, to obtain a final concentration of 10% (v/v)) resulting a final culture volume of 125 ml in the spinners and of 300 ml in the bioreactors. Three inoculums were used in the spinner vessel cultures, namely $1.2x10^5$, $2.4x10^5$ or $3.6x10^5$ cells/mL.

To perform cultures under environment-controlled conditions, commercially available bioreactors (Sartorius-Stedim Biostat Q-Plus system) with volume capacities between 200 and 500 ml were used. The agitation in the bioreactors was provided by marine impellers (in contrast to the paddle impellers in the spinners). The top of the bioreactors has multiple ports for different applications, such as sampling, addition or removal of medium or other supplements or solutions and placement of the temperature, pH and pO_2 sensors. Both pH and oxygen were controlled through the addition of gases via surface aeration. The pH of the culture was controlled at 7.4 with CO_2 and the pO_2 at 30% (of air saturation) with air and N_2. The temperature was kept at 37ºC by water recirculation in the vessel jacket controlled by a thermocirculator bath. Data acquisition and process control were performed using MFCS/Win Supervisory Control and Data Acquisition (SCADA)

software (B-Braun Biotech International GmbH, Melsungen, Germany). The inoculum used in the bioreactor cultures was $1.2x10^5$ cells/mL.

To avoid nutrient limitations and the accumulation of products of cellular metabolism that can be toxic to the cells, 50% of the culture medium was replaced every 4 days (in both spinner vessel and bioreactor cultures). To evaluate the effect of oxygen concentration in hepatocyte functionality and inducibility, three bioreactors were run in parallel using different oxygen levels (5%, 30% and 70 % of air saturation).

2.2.5. Sampling

Samples of cell spheroids and culture medium were collected at several time points to evaluate cell morphology and the synthesis/secretion and biotransformation capacities of hepatocytes. The samples were centrifuged at 50 g, and the supernatants were stored at -20ºC for albumin and urea quantification and at 4ºC for Lactate dehydrogenase (LDH) analysis as described by Racher (Racher 1998). Cell spheroid pellets were used for incubation with the proper substrates of ECOD and UGT to assess biotransformation capacities (described below in detail). Although some cellular death occurs mainly during the first days of culture (probed by LDH analysis), due to difficulties in disaggregating cell spheroids to assess the exact cell number, the ECOD and UGT activities are expressed by seeded hepatocytes (this is also a more accurate comparison between mono- and co-cultures since feeder cells have no hepatocyte specific functional capacities).

2.2.6. Fluorescent cell staining

To evaluate co-aggregation between hepatocytes and fibroblasts, both cells were stained separately (hepatocytes in green and fibroblasts in red), using a kit for general cell membrane labelling (PKH67 Fluorescent Cell Linker Mini Kit) following the instructions of the manufacturer. Briefly, cells were washed and then incubated

for 15 min in a dye at a concentration of $2.5x10^5$ cell/µl. Afterwards, cells were washed with culture medium and put together in culture.

2.2.7. Determination of albumin secretion and urea synthesis

Albumin secretion was measured by an enzyme-linked immunosorbent assay (NEPHRAT, Exocell, Philadelphia, USA). The urea synthesis rate was determined using a quantitative colorimetric kit (QuantiChromTM Urea Assay Kit, DIUR-500, ref DIUR-500; BioAssay Systems), according to the manufacturer's instructions.

2.2.8. 7-ethoxycoumarin-O-deethylase (ECOD) activity

7-ethoxycoumarin-O-deethylase (ECOD) activity was measured according to Gomez-Lechon et al. (1997) with slight modifications. Salicylamide (1.5 mM) was added to the medium to prevent conjugation of 7-hydroxy metabolites of 7-ethoxycoumarin. Therefore, hydrolyzation treatment with β-glucuronidase/arylsulfatase was avoided. The activity is expressed as µmol of 7-hydroxycoumarin formed per hour and per 10^6 hepatocytes inoculated. Day zero assay corresponds to the enzymatic activity of hepatocytes immediately after isolation. The activity refers to 7-hydroxycoumarin formed and is expressed as µmol/hour/10^6 hepatocytes.

2.2.9. Uridine diphosphate glucuronoltransferase (UGT) activity

Uridine diphosphate glucuronoltransferase (UGT) activity was determined by quantification of the substrate, 4-methylumbelliferone (4-MU), before and after cell incubation with the substrate. The procedure was performed according to Gomez-Lechon et al. (Gomez-Lechon *et al.* 1997) with slight modifications. Briefly, 100 µM solution of 4-MU in 0.01M PBS was incubated with cells for an hour at 37ºC. Samples fluorescence was analyzed at an excitation wavelength of 320 nm and emission of 450 nm. The 4-MU remaining concentration was determined based on a

standard curve generated in PBS spiked with 0, 1.5, 3, 6, 12.5, 25, 50 and 100 µM. The activity is expressed as µmol of 4-MU metabolized per hour and per 10^6 hepatocytes inoculated. Day zero assay corresponds to the enzymatic activity of hepatocytes immediately after isolation. The activity refers to 4-MU metabolized and is expressed as µmol/hour/10^6 hepatocytes.

2.2.10. Induction Assay

For the induction assay, the compound beta-Naphthoflavone (BNF), a CYP 1A2 inducer, was added to the bioreactor co-cultures at day 3, to a final concentration of 25 µM. Cultures were exposed to the inducer for a 72 h period. Samples were collected daily and enzymatic activity of ECOD was then measured as described above.

2.2.11. Statistics

Error bars correspond to the standard deviation of the average values. Data comparison between systems was performed using a two-way ANOVA test. Level of confidence was set at 0.05 for all tests, with p-values lower than 0.05 considered statistically significant. The albumin, urea, ECOD and UGT quantifications were performed in triplicate. The complete experiment (1 co-culture bioreactor, 1 co-culture spinner vessel, 1 mono-culture bioreactor, 1 monoculture spinner vessel, 1 2D co-culture and 1 2D mono-culture) was run twice (n=2).

3. RESULTS

3.1. Establishment of hepatocytes-fibroblasts 3D cultures

The aim of this work was to evaluate how 3D co-culture strategies impact hepatocytes functionality *in vitro*. For that purpose, freshly isolated rat hepatocytes were mixed with fibroblasts suspensions and inoculated under stirring conditions in small scale bioreactors or spinner vessels. The experimental set up is represented in **Figure 3.1**.

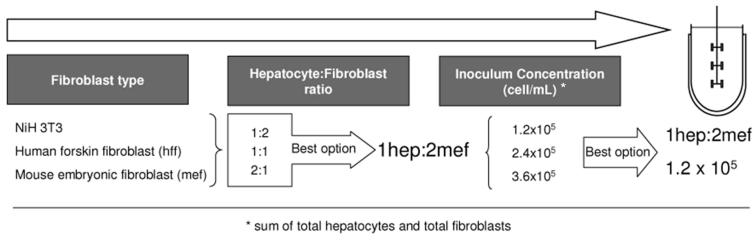

Figure 3.1.: Experimental set-up used to evaluate the effect of fibroblasts type, hepatocyte:fibroblast ratio and total cell inoculum concentration in hepatocytes functionality.

Three types of fibroblasts (mouse embryonic fibroblasts (mef), human foreskin fibroblasts (hff) and NIH 3T3 fibroblasts), as well as three ratios between hepatocytes and fibroblasts (1:1, 1:2 and 2:1) and different cell inoculum concentrations were first screened in 125 ml spinner vessels. These agitated culture vessels were used in this phase as they are hydrodynamically well characterized and their smaller working volumes allow more parallel experiments from the same hepatocytes batch preparation. Hepatocytes functionality was evaluated by measuring albumin and urea secretion and the activities of enzymes from phase I and phase II, namely ECOD and UGT, respectively. The best results were obtained when mouse embryonic fibroblasts were used as feeder cells, with a 1:2 ratio of hepatocytes to fibroblasts and an initial cell concentration of 1.2 $x10^5$ cell/mL (data not shown). Noteworthy, for all types of fibroblasts tested, the passage number (number of cell population doublings) was critical for the culture performance, with the best results being obtained for passages up to 5. Moreover, it could be observed that aggregates were more tightly packed when a higher number of fibroblasts per hepatocytes was used.

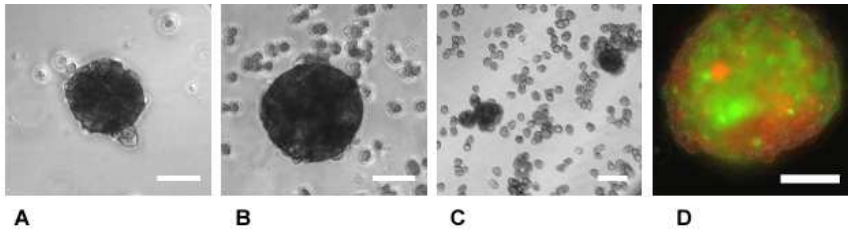

Figure 3.2.: Cell spheroids collected at day 3 from: (A) stirred tank bioreactors containing co-cultures of mouse embryonic fibroblasts (mef) and hepatocytes; (B) stirred tank bioreactors containing monocultures of hepatocytes; (C) spinner monocultures of hepatocytes used for comparison and evaluation of bioreactor performance; (D) co-culture with a ratio of 1hepatocyte:2mef; hepatocytes are stained in green and mef in red. Scale bars correspond to 100 µm.

As previously described for monotypic cultures of hepatocytes (Miranda *et al.* 2009), 3D cell spheroids could be detected in culture approximately 3 days after inoculation (**Fig. 3.2 A-C**). The increased difficulty in dissociating aggregates by trypsinization of mixed populations of hepatocytes and fibroblasts suggest a higher degree of cell-to-cell contact in co-culture aggregates when compared to monocultures. In co-cultures, the majority of the cells were assembled within the 3D spheroids (**Fig. 3.2 A**) while in hepatocyte monocultures single cells and cell doublets could be observed along culture time (**Fig. 3.2 B-C**). Evidence on 3D aggregates composed by both cells types can be seen on **Figure 3.2 D**, with hepatocytes staining in green and fibroblasts in red. The cell spheroids reach their maximum diameter by day 3 of culture. As cultured hepatocytes and inactivated fibroblasts lack the capacity of dividing, spheroid diameter depends mainly on the aggregation of more single cells (or small clusters of cells), which occurs essentially during the first three days of culture.

Our results show that the presence of fibroblast potentiate hepatocytes aggregation in both bioreactor and spinner vessels but more homogeneous populations of larger aggregates (ranging from 50-300 µm) are obtained in bioreactor cultures. Limitation on oxygen diffusion in spheroids bigger than 200 µm has been described in previous studies (Curcio *et al.* 2007), but this phenomenon is expected to be absent in our system, since the mechanic stirring facilitates oxygen

diffusion (as well as medium homogenization) both outside and inside the 3D cell structures, thereby avoiding the formation of necrotic cores (Moreira *et al.* 1995).

3.2. Stirred systems for the maintenance of 3D co-cultures: effect in hepatocytes functionality

Hepatocytes obtained from the same freshly isolated batch were cultured alone or with mouse embryonic fibroblasts in both bioreactor and spinner vessels. The total cell concentration at inoculation was equal for all cultures ($1.2x10^5$cells/mL), with the concentration of hepatocytes being three times lower in co-cultures (as a 1:2 of hepatocytes to fibroblasts ratio was used). These cultures were sampled during 21 days to evaluate the impact of both the presence of mouse embryonic fibroblasts and of a controlled environment on hepatocytes functionality. As a way to assess culture viability, the activity of LDH released to the supernatant by cell lysis was monitored over culture time (Racher 1998). This enzyme accumulated in the supernatant during the first five days of culture while cells adapt to the new environment but then their release decreased considerably until the end of the experiment.

Urea and albumin secretion rates were analyzed for the different culture systems. Urea secretion was observed during 21 days in all 3D cultures, without significant differences between them (data not shown). **Figure 3.3** shows albumin secretion rate per unit volume along culture time in the 3D culture systems.

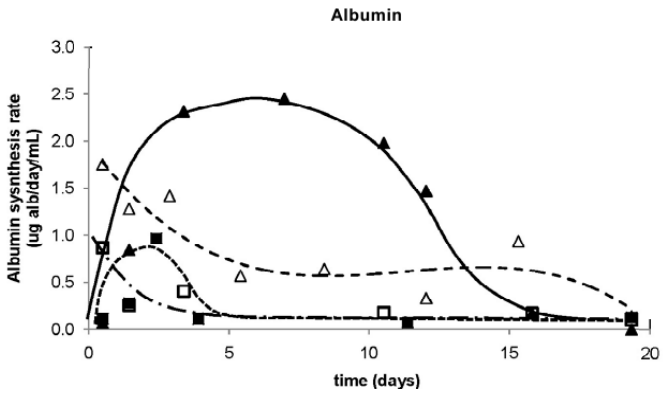

Figure 3.3.: Albumin secretion rate per unit volume along culture time in 3D stirred cultures. Full symbols correspond to bioreactor cultures and open symbols correspond to spinner cultures. Squares correspond to mono-cultures and triangles correspond to co-cultures. Solid and dashed lines correspond to trend lines of bioreactor and spinner cultures, respectively.

When co-cultured, hepatocytes exhibit significantly higher albumin production rates in both spinner and bioreactor vessels in comparison to the corresponding monotypic cultures ($p<0.05$). On the other hand, the controlled environment of the bioreactors allowed improved albumin secretion levels for the co-cultures but had no significant effect in the monotypic cultures. A constant decrease in albumin secretion rate along culture time is observed in spinner cultures whereas in the bioreactor cultures the secretion rate is low in the beginning but then increases, remaining high during an extended period in the bioreactor co-culture.

The biotransformation capacity of hepatocytes in the different culture systems was examined by measuring ECOD (Phase I enzyme) and UGT (Phase II enzyme) activities at different culture time points (**Fig. 3.4**).

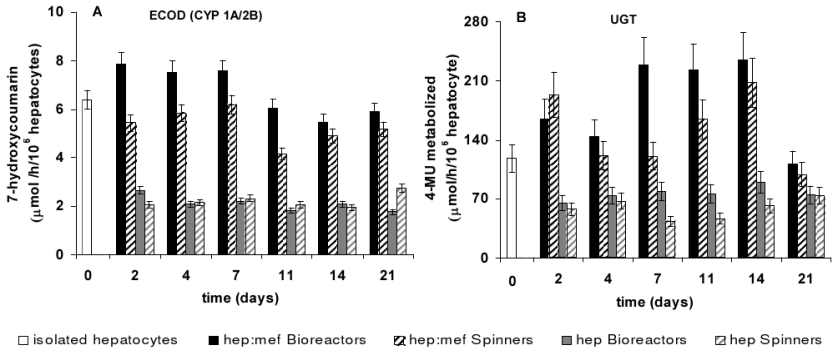

Figure 3.4.: Non-induced enzymatic activities along culture time in the different stirred 3D cultures (A) 7-ethoxycoumarin-O-deethylase (ECOD) and (B) uridine diphosphate glucuronoltransferase (UGT). Solid bars correspond to bioreactor cultures and dashed bars correspond to spinner cultures. Black bars correspond to co-cultures of hepatocytes and fibroblasts and grey bars correspond to hepatocyte monocultures. Day zero assay corresponds to the enzymatic activity of hepatocytes immediately after isolation.

Basal (non-induced) levels of the two enzymes remained at nearly the initial levels during the 21 days of culture for all culture systems and were significantly higher for hepatocytes co-cultured with fibroblasts. It is worthwhile to mention that these activities were either similar or even higher than the ones obtained immediately after hepatocytes isolation from the liver (**Fig. 3.4**). In general, liver-specific functions were significantly lower in 2D versus 3D cultures, as reported previously for both mono-cultures (Miranda *et al.* 2009) and co-cultures (Chia *et al.* 2005). A rapid loss of functionality was observed within 7 days for 2D mono-cultures. These values could be improved and extended until day 10 when hepatocytes were mixed with mef in 2D cultures.

3.3. Evaluation of hepatocytes response to CYP induction at different oxygen concentrations

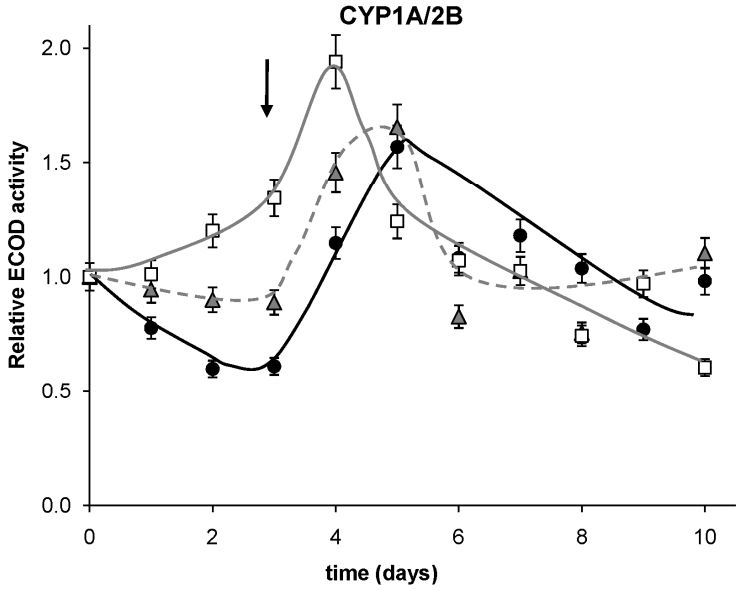

Figure 3.5.: Relative ECOD activity in 3D co-cultures performed in bioreactors operated at different oxygen levels, namely 5% (open squares, grey line), 30% (black circles, black line) and 70% (grey triangles, dashed line) of air saturation. The arrow indicates induction time with beta-Naphtoflavone.

The response of hepatocytes to the CYP1A inducer beta-Naphtoflavone (BNF) in different oxygen concentrations was examined for 3D co-cultures in bioreactors. Hepatocytes from the same freshly isolated batch were mixed with fibroblasts in a ratio 1:2 and used to inoculate three bioreactors: one with normoxic oxygen levels (30 % of air saturation) and the other two with extreme oxygen levels (5% and 70% of air saturation), in order to mimic hypoxia and high oxygen perfusion conditions. Induction was performed in these bioreactor co-cultures with BNF between days 3 (when the majority of cells are assembled in aggregates) and 6. ECOD activity was analyzed daily (during ten days) in all bioreactor cultures (**Fig. 3.5**). The ECOD activity ratio was maintained at inoculation levels in the high oxygen culture (70% of air saturation) but increased for cells exposed to low oxygen and

decreased for cells under normoxic conditions (**Fig. 3.5**). Differences in basal ratios of ECOD activity were observed among cultures: during the first three days, the ECOD activity ratio was maintained at inoculation levels in the high oxygen culture (70% of air saturation) but increased for cells exposed to low oxygen and decreased for cells under normoxic conditions. The induction response of hepatocytes to BNF exposure was observed for all cultures with the major fold increase in ECOD activity in the culture running at normoxic oxygen levels, 48 hours after induction.

The effect of dissolved oxygen in cellular glycolytic metabolism was also assessed. The ratio between lactate production and glucose consumption (Lac/Glc) was 0.5±0.07, 0.30±0.05 and 0.35±0.06 for cultures exposed to 5%, 30% and 70% of dissolved oxygen, respectively. The values obtained for cells exposed to normoxic and hyperoxic conditions were similar to the ratio Lac/Glc = 0.27 reported for hepatocytes cultured in a membrane aerated mini bioreactor (Schmitmeier et al 2006). The higher ratio observed for cells exposed to low oxygen levels (0.5; $p<0.05$) reflects a more anaerobic metabolism for this bioreactor co-culture.

4. DISCUSSION

The aim of this work was to develop a culture strategy for improving the overall hepatocyte functionalities *in vitro*. Three-dimensional (3D) cultures, where a cellular context "more-like" *in vivo* occurs, allows cells to integrate external signals, including those from cell-cell direct interaction and secretion/exchange of soluble factors and/or metabolites. Thus, strategically, we combined co-culturing approaches (by inoculating freshly isolated rat hepatocytes with fibroblasts) with scalable bioreactor technology (where sampling with minimal perturbation and long-term studies can be performed (Miranda *et al.* 2009)). 2D monolayer co-cultures previously described in several studies (e.g. (Bhatia *et al.* 1999; Cho *et al.* 2007)) were used as reference. We show that when co-cultured, both hepatocytes and fibroblasts self-assemble in 3D structures shortly after inoculation (**Fig. 3.2 D**) and that these cell spheroids can be maintained during long term in culture (up to 21

days). Moreover, we verified that fibroblast source and ratio to hepatocytes affect hepatocytes performance. From the three types of fibroblasts tested in the present study, the mouse embryonic were the type that allowed for the best improvement in hepatocyte functions. Many different fibroblasts from various organs and species have been shown to influence rat hepatocytes functionality (Bhatia *et al.* 1999). The specific mechanisms of interaction between cell types remain unclear.

Hepatocytes performance in the different culture conditions was assessed by monitoring several liver-specific functions, such as albumin and urea secretion. Drug detoxification capacity was also studied by evaluating biotransformation of xenobiotics, such as ECOD (phase I enzyme) and UGT (phase II enzyme) activities. We show that, not only the co-cultivation of hepatocytes with mouse embryonic fibroblasts (at a ratio of 1:2) improved their performance *in vitro* as compared to monotypic 2D and 3D cultures, but also that the effect of the feeder cells on hepatocytes functionality was potentiated when 3D approaches were used.

The long-term maintenance and inducibility of biotransformation enzymes in hepatic cultures have to be considered when determining drug metabolic pattern and predicting hepatotoxicity. Induction of CYP450 enzymes is clinically important as it can modify the metabolism of drugs, either potentiating their pharmacological action, diminishing their efficacy or producing unexpected hepatic side-effects. In this study, we evaluated the response of hepatic biotransformation enzymes to the action of BNF, a classical *in vivo* CYP1A inducer, in 3D co-cultures exposed to different oxygen levels (5%, 30% and 70% of air saturation). Dissolved oxygen has been described as a key factor to obtain long term functional hepatocyte cell cultures (Bader *et al.* 1999). There is still no consensus in the literature regarding the oxygen concentrations which reflect normoxic and hypoxic conditions in hepatocyte cultures (Fariss 1990; Martin *et al.* 2002; Nahmias *et al.* 2006). However, the levels in perivenous and periportal liver regions vary from 24% to 45% pO_2 (Jungermann and Kietzmann 1997). ECOD activity increased in the 3 bioreactor cultures after the induction insult (**Fig. 3.5**) with the higher increase observed at normoxic oxygen levels (30% of air saturation). It has been reported that hepatocytes tolerate well

hypoxic levels, due to their extraordinary capacity to satisfy energy requirements by anaerobic glycolysis (Bader *et al.* 1999). CYP forms are expressed and induced mainly downstream in the perivenous region where the cells have less oxygen available, but CYP1A inducibility in each zone depends on the inducer used, being BNF a stronger inducer of CYP1A on the periportal region (Oinonen and Lindros 1998). Our results follow this trend, i.e. before induction ECOD activity is higher in the lower oxygenated bioreactor (5% of air saturation) but after induction with BNF, cells exposed to high oxygen levels (30% and 70% of air saturation) showed improved inducibility. Some *in vitro* (Aninat *et al.* 2006; Josse *et al.* 2008) and *in vivo* (Diaz *et al.* 1990) studies have reported higher induction levels for lower basal CYP1A activities.

All together, our data show that co-culturing freshly isolated rat hepatocytes with mef cells, as 3D structures, in bioreactors, promotes hepatic cells maintenance for longer period of time (up to 21 days). By significantly enhancing and extending hepatocytes functionality, when compared with the traditional monotypic 2D monolayer cultures routinely used for toxicology studies, this *in vitro* model represent a promising and alternative tool for drug screening applications and drug development.

5. ACKNOWLEDGMENTS

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IV

HUMAN HEPATOCYTES IN 3D

This chapter is based on the following manuscript:

Human liver cell spheroids in extended perfusion bioreactor culture for repeated dose drug testing.
Tostões R, Leite SB, Serra M, Jensen J, Björquist P, Carrondo M, Brito C, Alves PM. (2011) Hepatology.

Sofia B. Leite performed the experimental work on the adaptation and optimization of the human hepatocytes to the stirring systems as well as all the work related with the co-cultures. Moreover, Sofia B. Leite was highly involved on the design of the experimental work of the study described in this Chapter.

ABSTRACT

Primary cultures of human hepatocyte spheroids are a promising *in vitro* model for long term studies of hepatic metabolism and cytotoxicity. The lack of robust methodologies to culture cell spheroids, a poor characterization of the human hepatocyte spheroids architecture and liver-specific functionality have hampered a widespread adoption of this 3D culture format. In this work, an automated perfusion bioreactor was used to obtain and maintain human hepatocyte spheroids. These spheroids were cultured for 3-4 weeks in serum-free conditions, sustaining their phase I enzyme expression and permitting repeated induction during long culture times; the rate of albumin and urea synthesis, as well as phase I and II drug metabolizing enzymes' gene expression and activity of the spheroid hepatocyte cultures presented reproducible profiles, despite the basal inter-donor variability (n=3 donors). Immunofluorescence microscopy of human hepatocyte spheroids after 3-4 weeks of long term culture confirmed the presence of the liver-specific markers hepatocyte nuclear factor 4α, albumin, cytokeratin 18 and cytochrome P450 3A. Moreover, immunostaining of the atypical protein kinase C apical marker, as well as the excretion of a fluorescent dye, evidenced that these spheroids spontaneously assemble a functional bile canaliculi network extending from the surface to the interior of the spheroids after 3-4 weeks of culture. Conclusion: Perfusion bioreactor cultures of primary human hepatocyte spheroids maintain a liver-specific activity and architecture and are thus suitable for drug testing in a long term, repeated dose format.

CONTENTS

1. INTRODUCTION

The liver-specific functions of hepatocytes, such as albumin secretion or drug metabolizing activity, are rapidly downregulated during *in vitro* primary cultures, limiting their use for drug development and toxicity tests (Gomez-Lechon *et al.* 2003). For such assays, the current gold standard for long term human hepatocyte culture is the collagen sandwich *in vitro* model (Hewitt *et al.* 2007). The overlaying collagen layer increases cell-cell and cell-matrix contacts, providing a more 3D-like architecture than a monolayer culture. For rat hepatocyte spheroids, where cell-cell interactions are maximized, liver-specific functions (Wu *et al.* 1999; Miranda *et al.* 2009) and multicellular architecture (Abu-Absi *et al.* 2002; Dvir-Ginzberg *et al.* 2004) are increased, when compared to monolayer cultures.

The use of microfluidic devices for primary cultures of hepatocytes is a promising approach to enable high throughput screening in drug development (Goral *et al.* 2010; Nishimura *et al.* 2010). However, the down scaling enabled by these technologies makes the culture environment harder to be controlled and limits the application of microfluidics for long term primary cultures of hepatocytes. In fact, the most useful applications of micro technologies for such cultures couple either microfluidic perfusion or co-culture micropatterning to 12 (Domansky *et al.* 2010) or 24 well plate culture plates (Khetani and Bhatia 2008), respectively; still, these technologies do not enable a physiologically relevant long-term culture of primary hepatocytes.

Bioartificial liver devices (BAL) using human hepatocytes are often built in hollow fiber formats; this configuration is likely among the best options for maintaining cultures of large numbers of hepatocytes for prolonged culture periods (Carpentier *et al.* 2009); recently, one of such devices has also been scaled down and adapted to drug testing, using human liver cells (Zeilinger *et al.* 2011). The configuration of these bioreactors enables the formation of a liver-like hepatic mass throughout the culture time, making them long-term end point assays; nevertheless,

these bioreactors were not designed for high throughput screening and do not allow sampling of the cellular mass throughout the culture period.

CYP450 expression is downregulated during *in vitro* primary culture of hepatocytes; these enzymes are fundamental to xenobiotic metabolization studies, namely in drug development, and the orphan receptor-mediated induction of their mRNA synthesis is one of the most important parameters to be assessed in such tests (LeCluyse 2001). After these enzymes have oxidized a given xenobiotic, the compound is further conjugated with polar groups by phase II enzymes and secreted in the bile canaliculi by phase III enzymes. Thus, a long-term hepatocyte culture system must not only maintain CYP450 basal expression but also to enable their *de novo* mRNA synthesis upon exposure to prototypical CYP450 inducers, while maintaining the remaining phase II and III activities. Since phase III activity depends on the transport of phase II metabolites through the apical membrane, the presence of bile canaliculi is also necessary for a thorough assessment of drug metabolizing activity in primary cultures of hepatocytes.

This work focuses on the use of perfusion stirred tank bioreactors for primary cultures of human hepatocyte as spheroids. When cultured in a bioreactor with essentially convective mass transfer and environmental control, the hepatocytes experience much smaller changes in pH, dissolved oxygen (DO) and culture medium composition than any culture system with a constant atmosphere and discrete medium exchanges. The pH and DO are controlled by CO_2 and N_2 injection through the reactor headspace, respectively, whereas the continuous addition of nutrients and removal of metabolic by-products is ensured by the automated perfusion system (Tostoes *et al.* 2011); the good mixing minimizes the gradients of these soluble factors in the culture bulk. The results indicate that this bioreactor system of primary culture of human hepatocyte spheroids enables the robust formation of hepatic-like micro-tissue units that can be repeatedly induced in long-term periods.

2. MATERIALS AND METHODS

2.1. Primary cultures of human hepatocytes

Human liver samples originating from patients undergoing liver resection (Table 4.1), were obtained from Sahlgrenska´s Hospital (Gothenburg, Sweden) upon written consent in agreement with ethical approval and from the patient signed informed consent agreement. The human liver samples were isolated by a two-step EDTA/collagenase type IV perfusion, followed by slow-speed centrifugation to reduce red blood cell content. Cells were resupended in Williams E medium, and stored over night (shipped over night) at 4ºC in Williams E medium before use. At the beginning of the experiments (upon arrival) cell viability was higher than 80% as determined by trypan blue exclusion, using a Fuchs-Rosenthal counting chamber.

Table 4.1: Donor Information

Donor	Gender	Age	Weight	Ethnicity
0	M	73	89	Caucasian
A	F	42	78	Caucasian
B	M	57	80	Not known
C	F	73	70	Not known
D	F	30	56	Caucasian
E	F	39	71	Caucasian

2.2. Perfusion Bioreactor Culture

Primary cultures of human hepatocytes were performed in Williams' E medium supplemented with 1% Glutamax, 1% Penicilin/Streptomycin (all from GIBCO/Invitrogen) and Hepatocyte Culture Medium (HCM) Single Quots (Lonza, Walkersville, MD) (WE). The bioreactor cultures were inoculated at a cell concentration of $0.2x10^6$ viable hepatocytes/ml. To promote cell aggregation into spheroids WE complete medium was supplemented with 10% FBS; cells were cultured in this medium for 72h and in serum free conditions afterwards (**Fig. 4.1**); the hepatocyte culture viability after aggregation was always above 90%, as assessed by the Trypan Blue exclusion method. The initial bioreactor working volume (**V**) was 300 ml and the perfusion rate (**F**) was set to 60 ml/day; this rate was adjusted throughout the culture time (as the culture volume decreased due to sampling) to maintain a dilution rate (**D**) of 0.2 day^{-1} (i.e., 20% medium exchange per day; **D=F/V**). The bioreactor (Sartorius-Stedim Biostat Q-Plus system) cultures were controlled at 37ºC, pH=7.4 and DO at 30% air saturation (approximately 60 µM of O_2 or 6.3% oxygen in a controlled atmosphere incubator, assuming an efficient mass transfer to the culture bulk).

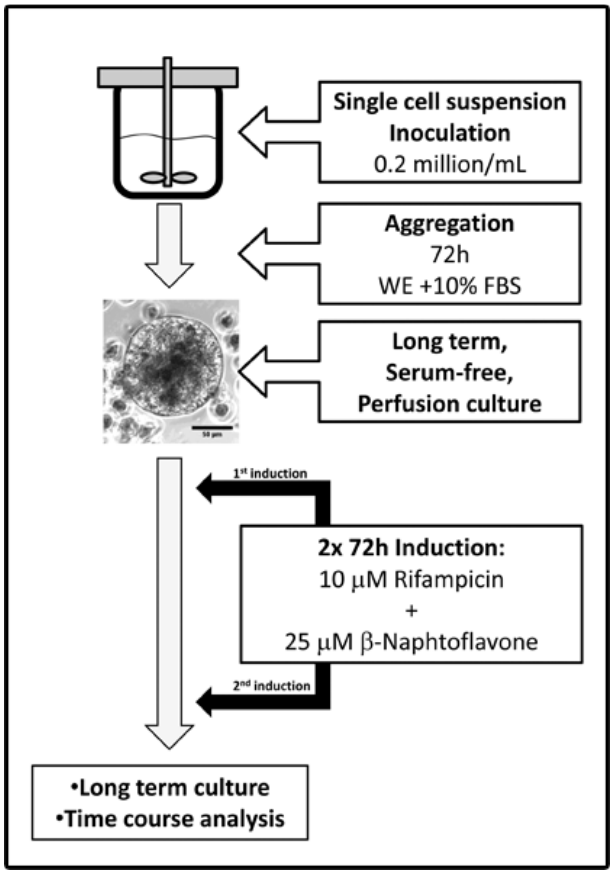

Figure 4.1.: Experimental design of the induction of the CYP450 enzymes in primary cultures of hepatocyte spheroids in the bioreactor.

2.3. Hepatocyte CYP450 enzyme inductions

Inductions of the hepatocyte spheroids' CYP450 enzymes were performed as depicted in **Figure 4.1**. Briefly, inductions in bioreactors were started by adding Rifampicin (Rif) and β-Naphtoflavone (BNF) at concentrations of 10 and 25 μM, respectively. The bioreactors were perfused with culture medium, containing the same concentrations of both inducers, at a dilution rate of 0.5 day^{-1} (2.5 times the dilution rate used for normal perfusion culture), for 72h; after this period, culture medium was fully exchanged to ensure a complete removal of the induction medium.

This induction procedure was performed twice, at day 3 and 2-4 weeks later, for all donors (**Fig. 4.1**).

2.4. Cell concentration determination

Spheroids were digested with 0.05% Trypsin/EDTA (GIBCO) and the resulting single cell suspension viability was assessed by the Trypan Blue exclusion method. Cell counting was performed using a Fuchs Rosenthal counting chamber.

2.5. Hepatocyte spheroids visualization and measurement

Hepatocyte spheroids were visualized by phase contrast microscopy (Leica Microsystems GmbH, Wetzlar, Germany) and their average diameter ($\mathbf{d_{ave}}$) was determined by a geometric mean of three diameters per spheroid using the equation $\mathbf{d_{ave}=(d_1 \times d_2 \times d_3)^{1/3}}$; spheroid diameters were measured using the ImageJ software. Diameter distribution plots were done using the GraphPad Prism software (La Jolla, CA 92037 USA).

2.6. Determination of albumin and urea synthesis

The secretion of albumin from hepatocytes was measured by an enzyme-linked immunosorbent assay (ELISA) using the Exocell Albuwell albumin test kit (Philadelphia, PA, USA). The assay was performed according to the manufacturer's description. The urea synthesis rate was determined using a quantitative colorimetric urea kit (QuantiChromTM Urea Assay Kit, DIUR-500, BioAssay Systems), according to the manufacturer's instructions. The albumin and urea specific synthesis rates were calculated according to the general mass balance equation for a continuous system: $\mathbf{q=(\Delta C/\Delta t - D \times (C_{in} - C_{out}))/ [X]_{V\ average}}$ where **q** is the specific synthesis rate, $\mathbf{\Delta C/\Delta t}$ is the rate of change of the metabolite (either urea or albumin) in the supernatant, **D** is the dilution rate (0.2 day^{-1}), $\mathbf{C_{in}}$ and $\mathbf{C_{out}}$ are the inlet and outlet concentrations of the metabolite and $\mathbf{[X]_{V\ average}}$ is the average viable cell

concentration during **Δt**. The results were expressed as μg/10^6 cells/day at the indicated time points.

2.7. CYP450 activity Measurement

7-Ethoxycoumarin O-deethylation (ECOD) activity was measured using the method described in (Castell and Gómez-Lechón 1997) with slight modifications. Briefly, Salicylamide (1.5 mM), was added to the medium to prevent conjugation of 7-hydroxy metabolites (7-HC) of 7-ethoxycoumarin. The activity is measured by the rate of formation of 7-hydroxycoumarin (Umbelliferone) in nmol/10^6 cells/day.

2.8. qRT-PCR

Hepatocyte spheroids were collected from bioreactor cultures at different time points and stored at -20ºC with RNAprotect Cell Reagent (Qiagen, Valencia, CA). Later total RNA was extracted using RNEasy Plus Mini Kit (Qiagen) according to the manufacturer´s instructions. Reverse transcription was performed using 0.6ug of total RNA in a final volume of 20 μl reaction mix using High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). Real time PCR was performed using ready-to-use TaqMan Gene Expression Assays (applied Biosystems) according to the manufacturer´s instructions. GAPDH was used as endogenous control.

2.9. Structural arrangement of the cell spheroids

2.9.1.Whole mount Immunofluorescence microscopy

Hepatocyte spheroids were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS for 1 hour at room temperature (RT), blocked overnight (O/N) at 4ºC in 0.1 % Triton X-100 (Sigma-Aldrich) and 0.2% fish skin gelatin solution in PBS and subsequently incubated with primary antibodies diluted (1:100) in 0.125% fish skin gelatine in PBS for 2 days at 4°C. Cells were washed three times with PBS and the secondary

antibodies (diluted 1:500 in 0.125% fish skin gelatine in PBS) were applied to the cells overnight at 4ºC in the dark. After three washes with PBS, the samples were mounted in Prolong gold anti-fade containing 4,6-diamidino-2-phenylindole (DAPI). Cells were visualized using point scanning (SP5, Leica Microsystems GmbH, Wetzlar, Germany) or spinning disk (Andor Technology, Belfast, Northern Ireland) confocal microscopy.

2.9.2. CryoSection immunofluorescence microscopy

Hepatocyte spheroids were frozen in O.C.T.™ Tissue Tek (Sakura Finetek Europe, NL) and sectioned in 10 µm thick slices onto glass coverslips. These coverslips were blocked for 10 minutes at RT in 0.1 % Triton X-100 (Sigma-Aldrich) and 0.2% fish skin gelatin solution in PBS and subsequently incubated with primary antibodies diluted (1:100) in 0.125% fish skin gelatine in PBS for 2 hours at 4°C. Cells were washed three times with PBS and the secondary antibodies (diluted 1:500 in 0.125% fish skin gelatine in PBS) were applied to the cells for 1h at 4ºC in the dark. After three washes in PBS, the samples were mounted in Prolong gold anti-fade containing DAPI. Cells were visualized using a Leica DMI 6000 epifluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

2.9.3. Antibodies for confocal immunofluorescence microscopy

Primary antibodies used were: goat anti-albumin, mouse anti-HNF4α (Abcam, Cambridge, UK), goat anti-CYP450 3A, mouse anti-aPKC (Santa Cruz, CA. 95060), FITC conjugated anti-Cytokeratin 18 (Sigma Aldrich, St. Louis, MO), Alexa 488 conjugated Phalloidin (Molecular Probes, Eugene, OR). Secundary antibodies used were anti-mouse Alexa Fluor 488, anti-goat Alexa Fluor 594 (Molecular Probes, Eugene, OR) and anti-mouse Cy5 (Abcam, Cambridge, UK).

2.9.4. Bile canaliculi functionality

Human hepatocyte spheroids were collected from the reactor after 2 weeks of culture, washed with PBS and incubated for 10 minutes in 2 µg/ml of 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (CDFDA; Molecular Probes, Eugene, OR) in Williams E medium. Afterwards, spheroids were washed for 3 times with PBS and imaged in by confocal spinning disk microscopy (Andor Technology, Belfast, Northern Ireland).

2.10. Co-Culture

Preliminary studies of the influence of human forskin fibroblasts (hff) in the functionalities of human hepatocytes were performed in the bioreactor with no perfusion. In this study, were used cells from Donor E. Cultures were performed as described in Chapter 3 (Leite *et al.* 2011). Briefly, hff (from ATCC) were cultured in monolayer culture flasks until reach confluence. Before inoculation of the bioreactor, hff were growth arrested by adding 100 µM of Mytomicin for 3 hours into the culture medium and then washed away. For bioreactor innoculation single-cell suspensions of hepatocytes and fibroblasts were mixed together with a proportion of 1hepatocyte:2hff. Bioreactor was run at a final concentration of $0.12x10^6$ cells/ml. A Mono-culture bioreactor was performed in parallel as control. Both bioreactor were performed as described in section 2.2., but instead of using perfusion system, culture medium was exchanged every 4 days.

2.10.1. Hepatic functionalities in Co-Culture

For functionality assessment it was quantified the albumin in the culture medium and the ECOD activity of the cells as described before (sections 2.5 and 2.6). The quantified values are expressed in μg albumin/10^6 hepatocytes inoculated and nmol of Umbeliferone formed/h/10^6 hepatocytes inoculated respectively.

2.11. Statistical analysis

Unless otherwise stated, all results were subject to an ANOVA single factor analysis, with α=0.05, using Microsoft Excell's data analysis toolpack; p values are presented for statistically significant results ($p<0.05$).

3. RESULTS

3.1. Hepatocyte spheroid size distribution, viability and liver-specific synthesis time-course profiles in bioreactor cultures.

The hepatocyte spheroid diameter is a critical variable for the maintenance of viability (Curcio *et al.* 2007) and hepatic phenotype (Glicklis *et al.* 2004) in primary rat hepatocyte cultures. Thus, the average spheroid diameters in bioreactor cultures were measured based on phase-contrast images after 1 and 2 weeks of culture, as depicted in **Figures 4.2 a** and **4.2 b** (Donor C), yielding a size distribution plot as shown in **Figure 4.2 c**. On average, the spheroid diameters were 65 ± 7 μm after 1 week and 81 ± 4 μm (value ± standard error of the mean) after 2 weeks, for the 3 different donors (**Fig. 4.2 d**), and the viability of the primary bioreactor cultures of hepatocyte spheroids was maintained above 70% of the inoculated viable hepatocytes without major cell death during culture time (**Fig. 4.2 d**).

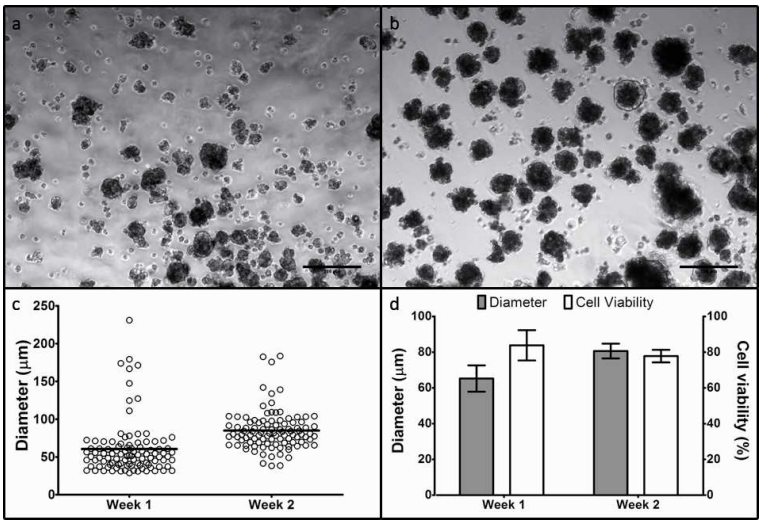

Figure 4.2.: Hepatocyte spheroid diameter distribution and cell viability. Bright field pictures of human hepatocyte spheroids in the first (a) and second (b) week of culture show an increase in average diameter, as quantified in c (Donor C, n= 100 spheroids; bars=200 µm). The average diameter of the hepatocyte spheroids and the average cell culture viability (in % of viable inoculated hepatocytes) are depicted in d. Bars in d represent standard deviations of n= 3 donors.

Urea and albumin secretion, two liver-specific functions essential for ammonia detoxification and to maintain the blood osmotic pressure, respectively, were analyzed during the hepatocyte spheroid bioreactor culture; **Figure 4.3** shows that the time course profiles for urea (a) and albumin (b) synthesis were comparable and reproducible among the 3 donors. The specific albumin production rate increases steadily along the 15 days culture time, whereas urea production decreases from the onset of the culture, reaching a steady state after 1 week of bioreactor culture. For both liver-specific activities, as much as 10 fold inter-donor variability was observed.

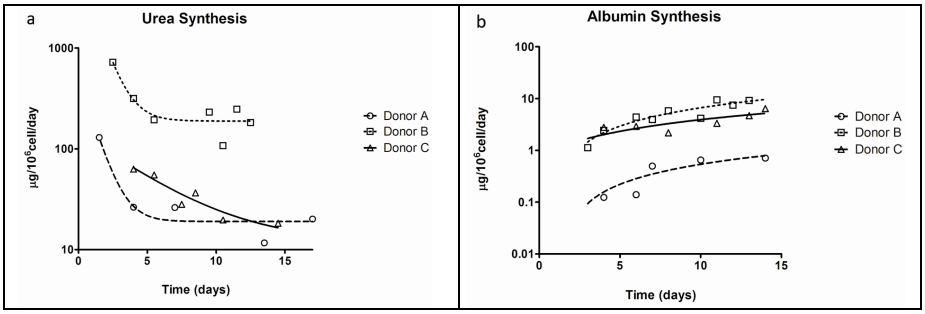

Figure 4.3.: Urea and albumin synthesis in bioreactor hepatocyte spheroid cultures. Bioreactor cultures of human hepatocyte spheroids show reproducible profiles for urea (a) and albumin (b) synthesis for donors A (○), B (□) and C (△).

3.2. Phase I and II enzyme expression and activity in bioreactor cultures.

Hepatic drug metabolization typically involves phase I monooxygenase activity (CYP450 activity) followed by phase II conjugation activity and the transport of hydrophilic metabolites by phase III transporters. The gene expression for 3 CYP450 isoforms (1A2, 2C9 and 3A4) and 2 conjugation enzymes (GSTA1 and UGT2B7) was measured, for the 3 donors, after spheroid aggregation (i.e., at day 3; **Fig. 4.4 a**) and during the remaining culture time (**Fig. 4.4 b-d**); in addition, 2 prototypical inducers, Rifampicin and β-Naphtoflavone, were added to the cultures in a repeated dose (**Fig. 4.4 b-d**, dashed boxes).

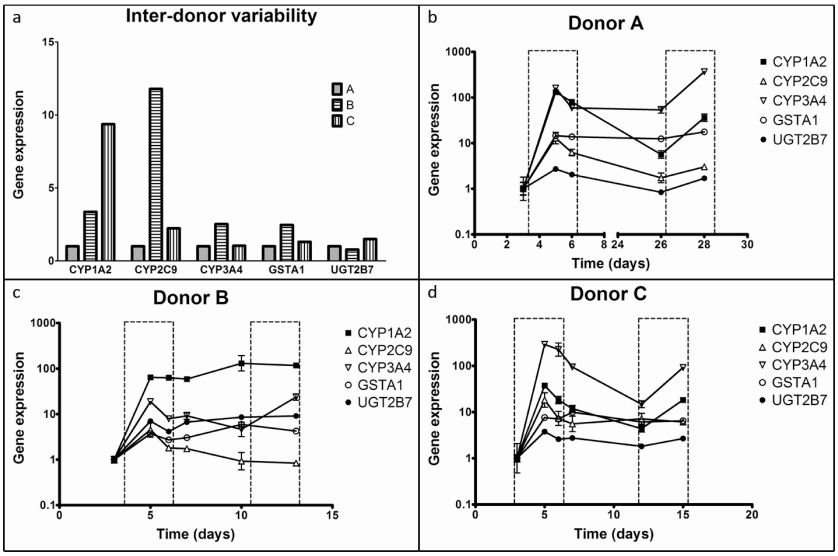

Figure 4.4.: Inter-donor variability and time course profiles of phase I and II enzymes in human hepatocyte spheroids bioreactor cultures. The gene expression of the CYP450/phase I enzymes 1A2, 2C9 and 3A4, phase II enzymes GSTA1 and UGT2B7 was measured at day 3 (when spheroids were formed) and normalized to Donor A values (a); the same genes' expression was monitored for the remaining culture time for donors A (b), B (c) and C (d). The gene expression values in (b,c,d) were normalized to the respective gene expression at day 3. CYP450 1A2 (■), 2C9 (△), 3A4 (▽); phase II enzymes GSTA1 (○) and UGT2B7 (●). The dashed areas represent the induction period, when spheroids were exposed to both Rif (10 M) and BNF (25 M). Bars represent standard deviations of 3 different samples from bioreactor.

Differences between donors for the gene expression of these drug metabolizing enzymes may vary 10-100 fold (Hewitt *et al.* 2006); this inter-donor variability is depicted in **Figure 4.4 a**, where gene expression for each enzyme was normalized to donor A values. Thus, normalization of relative gene expression to day 3 is required to compare the temporal evolution of the system between donors, as depicted in **Figures 4.4 b-d**. An increase in the mRNA expression of drug metabolizing enzymes has been observed at day 5, after the addition of the inducer cocktail (at day 3), for all donors; these expression levels decrease after the first induction period, for donors A and C, whereas for donor B not all enzymes had a reduction in expression level. The second induction period was performed 72h before the end of the cultures (between weeks 2 and 4), while for donors A and C cultures there was a general upregulation of gene expression, donor B culture only showed an increase in CYP3A4 mRNA after this induction.

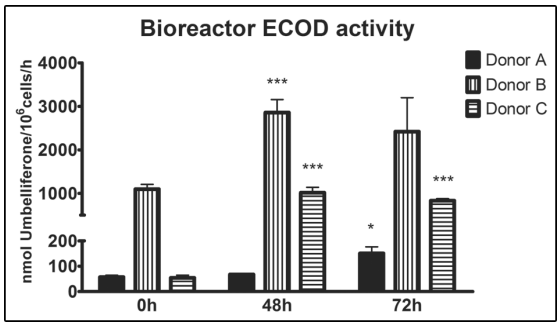

Figure 4.5.: Induction of ECOD activity in bioreactor (see also Figure 4.1 for methodology). The ECOD activity of the bioreactor cultures was assessed after 48 and 72h of RIF and BNF exposure. Bars represent the standard error of the mean of at least 3 independent cell-based measurements; ANOVA was performed by comparing 0h (basal) and 48 or 72h values, for each donor. *, $p<0.05$; ***, $p<0.001$.

The CYP450 activity of the hepatocyte spheroids was measured by the metabolization of 7-Ethoxycoumarin to 7-Hydroxycoumarin, a reaction mainly catalyzed by the CYP1A family, even though CYP2 and CYP3 s are also involved in this deethylation reaction (Waxman and Chang 2006). Samples were collected from the bioreactors at 48 and 72h, during the first induction period (**Fig. 4.5**); the ECOD activity of all the 3 donors significantly increased either by 48h (Donor B), 72h (Donor A) or at both time points (Donor C). These fold-increases ranged from 2.6 for donors A (72h) and B (48h) to 19 and 15 for Donor C (at 48 and 72h, respectively).

3.3. Liver-specific markers and structural polarity in bioreactor cultured spheroids.

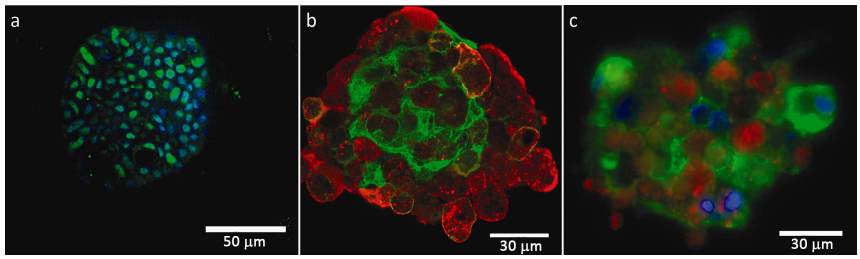

Figure 4.6.: Immunofluorescence microscopy of liver-specific antigens in human hepatocyte spheroids after 2 weeks of bioreactor culture. (a) HNF4α (green) co-localizes with the nuclear DAPI staining (blue); (b) albumin (red), Cytokeratin 18 (green); (c) CYP450 isoform 3A (red), Cytokeratin 18 (green) and nuclei (DAPI,blue). Samples for panels a and b were prepared in whole-mount, while the samples for panel c were prepared as 10 µm thick cryosections.

Human hepatocyte spheroids cultured in fully controlled bioreactors, from donors A and C, were analyzed by immunofluorescence microscopy to assess the presence of HNF4α, Cytokeratin18, Albumin, CYP450 3A and polarity markers inside such spheroids. For donor A, both Albumin (**Fig. 4.6 b**, red) and CYP450 3A (**Fig. 4.6 c**, red) were still detectable at 30 days of culture, as well as Cytokeratin 18 (**Fig. 4.6 b** and **c**, green); the donor C culture stained positive for HNF4α after 20 days in culture (**Fig. 4.6 a**).

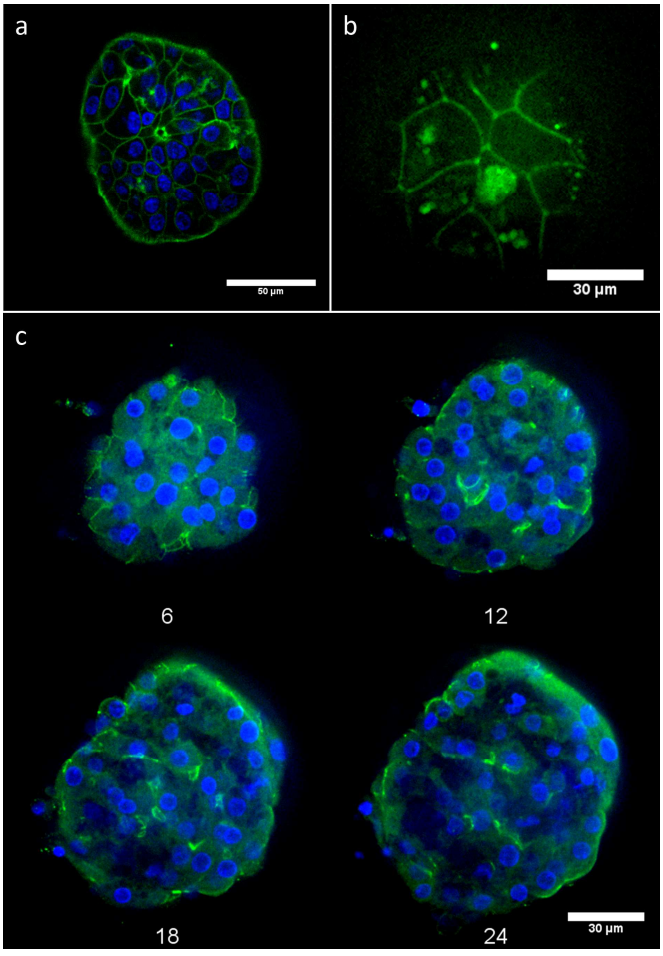

Figure 4.7.: Fluorescence microscopy of structural and polarity markers and bile canaliculi function in human hepatocyte spheroids after 2 weeks of bioreactor culture. (a) F-actin (green) localizes to cell membranes and is enriched in bile-canaliculi-like structures (arrow); (b) transport of CDFDA to the apical (canalicular) domain of the hepatocytes shows an extensive and interconnected canaliculi network. (c) Confocal z-sections (numbers represent the distance from the spheroid surface, in µm) of a hepatocyte spheroid stained for aPKC (green) and nuclei (DAPI, blue) shows several bile canaliculi-like channels which extend to the interior of the spheroid.

In donor D hepatocyte spheroids, actin (phalloidin) staining (**Fig. 4.7 a**, green) shows an absence of stress fibers, with most of these filaments localizing at the intercellular borders; furthermore, an actin enrichment can be seen, in some cell-cell contacts, which forms canaliculi-like structures (**Fig. 4.7 a**), very similar to the *in vivo* liver tissue architecture (Dunn *et al.* 1991). The establishment of *de novo* polarity is more obvious when Donor C hepatocyte spheroids were immunostained for atypical Protein Kinase C (aPKC), a kinase associated with the apical domain of epithelial cells (**Fig. 4.7 c**, green): a series of 24 µm confocal Z-stacks shows a bile canaliculi network which extends to the inner part of the spheroid (**Fig. 4.7 c**). The functionality of these channels was assessed by imaging the excretion of CDFDA into the canaliculi after being metabolized by the hepatocytes' intracellular esterases (**Fig. 4.7 b**), in two week old spheroid cultures of Donor D.

3.4. Effect of feeder cells in the hepatic functionalities

Following the strategy of work from Chapter 3, and on the advantage of coupling the use of feeder cells to the bioreactor approach to improve human hepatocyte specific activities (Khetani and Bhatia 2008) a preliminary study with cells from Donor E was performed in order to check the effect of fibroblasts on hepatic specific functions. Results are shown on **Figure 4.8**.

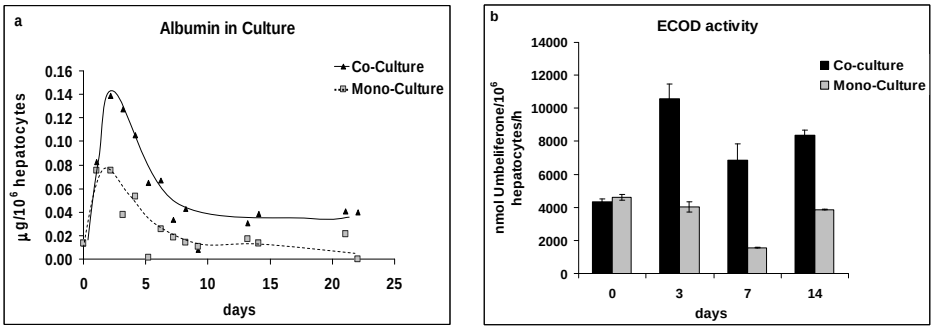

Figure 4.8.: Albumin quantification (a) and ECOD activity (b) of human hepatocytes (Donor E) in Mono- and Co-culture with human fibroblasts (hff). The presence of feeder cells (▲) seem to improve the basal functionality of Human hepatocytes (□).

On **Figure 4.8a** it can be seen the levels of albumin in the medium in Mono- and Co- Cultures, run in parallel with same stratagem. It can be seen that after a first increase of the levels in the culture medium during the first week, the albumin accumulated reaches a lower steady state, that is close to the values of freshly isolated cells, in the case of the Mono-culture, and 2-3 times higher in the case of the Co-culture. Regarding biotransformation (**Fig. 4.8 b**) assessment, it can be seen that the presence of hff cells improved the ECOD metabolism 2-4 times on first, second and third weeks.

4. DISCUSSION

In this work, a perfused bioreactor system for long term maintenance of primary cultures of human hepatocyte spheroids was established and tested. In this system, hepatocyte spheroids reproducibly recapitulated *in vivo* hepatic functions and structure, despite inter-donor variability. We hypothesize that these reproducible time-course profiles were made possible due to the tight control of critical environmental variables at physiological values, such as pH and oxygen levels. mRNA expression of CYP450 (phase I), GSTA1 and UGT2B7 (phase II) was maintained up to 4 weeks and increased when the cultures were exposed to the prototypical CYP450 inducers Rifampicin and β-Naphtoflavone, in repeated dose. The phase I ECOD activity of such cultures also responded to such inducers, showing the system's potential for more informative time course experiments. The spheroid's inner structure resembled the liver architecture, with functional bile canaliculi-like structures and liver-specific markers. Such a system constitutes an ideal long-term culture platform for analyzing hepatic function for drug development tests.

The formation of hepatocyte spheroids was performed during the first 72 hours of culture; phase contrast microscopy data of this period (data not shown) suggests an initial 24h period of cell clustering, when small aggregates (40-50 μm)

are formed; these clusters grow in size during the following 2 days (i.e., until 72h), as previously reported for primary cultures of rat hepatocyte spheroids (Wu *et al.* 1996).

The influence of oxygen concentration in primary cultures of hepatocytes has been the subject of several publications (Foy *et al.* 1994; Curcio *et al.* 2007; Kidambi *et al.* 2009; Leite *et al.* 2011) and the conclusions from these studies are not easily comparable. This is mainly due to the different culture systems used, i.e., static systems (Kidambi *et al.* 2009) need higher oxygen concentrations for hepatocyte culture because the mass transfer relies on diffusion; on the other hand, stirred systems, such as the bioreactor described herein, have convective mass transfer and nearly homogeneous DO concentrations in the culture bulk. Thus, the bioreactor culture oxygen concentration used in this work (30% of air saturation in culture medium, i.e., 60 μM) is in the interval between the known periportal and pericentral oxygen concentration in the rat liver, 90 and 45 μM, respectively (Jungermann and Kietzmann 1997).

The bulk aggregation of hepatocytes into multicellular spheroids depends on parameters such as the agitation type, vessel geometry (Khaoustov *et al.* 1999; Brophy *et al.* 2009; Miranda *et al.* 2009) and cell inoculum (Miranda *et al.* 2009). Brophy and colleagues (Brophy *et al.* 2009) have show that rat hepatocyte spheroids could be obtained from single cells by rocking motion with a higher yield of spheroids (85%), when compared to rotational motion (54%). However, this rotational motion was based on shake flask cultures and previous work by the Hu group (Wu *et al.* 1996) had shown that spinner vessels could yield rat hepatocyte spheroids with an 80% efficiency in the incorporation of single cells into spheroids, after 72h. The differences between the fluid dynamics in orbitally shaken flasks and spinner vessels or the different cell inoculum used (1 and 0.3 million hepatocytes per ml, respectively) can explain the difference in both publications. In the work described herein, the previous knowledge from our group concerning the aggregation of rat hepatocytes (Miranda *et al.* 2009; Leite *et al.* 2011; Tostoes *et al.* 2011) was adapted to the formation of multicellular spheroids in primary cultures of human hepatocytes.

For these cultures the control of spheroid size becomes critical to avoid putative nutrient diffusion limitations; in the system described herein, hepatocyte spheroids had an average size of 81 μm (**Fig. 4.2 d**) and the number of spheroids with a diameter larger than 200 μm was less than 0.4% of the total population (n=3 donors). From previously published studies, it is know that rat hepatocyte spheroids with diameters of 100 μm yield a higher albumin production rate than larger ones (Glicklis *et al.* 2004), whereas spheroids of up to 200 μm diameter have been shown not to be subject to nutrient (namely oxygen) limitations (Curcio *et al.* 2007). Since the aggregation process here described reproducibly yields spheroids with an average 81 μm diameter, it is not expected that the hepatocytes within these spheroids are subject to any significant mass transfer limitations.

The data obtained for liver-specific activities (urea and albumin production, **Fig. 4.3**; ECOD activity, **Fig. 4.5**) and gene expression (CYP450 and phase II enzymes, **Fig. 4.4**) confirm the inter-donor variability, which has been thoroughly described for primary human hepatocyte cultures and is a direct reflection of *in vivo* variability (Ponsoda *et al.* 2001; Gomez-Lechon *et al.* 2003); however, the tight control of critical variables by the perfusion bioreactor system, coupled to an easy cell sampling system, allowed reproducible liver-specific profiles to be obtained, despite inter-donor variability; the use of serum-free media after aggregation is also a likely cause for such a reproducible behavior, since serum is known to downregulate both albumin synthesis and CYP450 activity of primary cultures of human hepatocytes (Kidambi *et al.* 2009). The profiles of urea secretion rate for the 3 donors have a significant decrease from the beginning of the cultures. This reduction in urea productivity has been observed by Zeillinger and colleagues (Schmelzer *et al.* 2009; Zeilinger *et al.* 2011) in a perfusion hollow fiber bioreactor and may be related to the lower oxygen concentrations inside the spheroids: retrograde liver perfusion experiments in rats have shown that lower oxygen concentrations partially inhibit the periportal urea synthesis (Katz 1992).

The primary cultures of human hepatocytes in the perfusion bioreactor were inducible for the entire long-term period. The co-administration of Rif and BNF ensured increase in mRNA synthesis for CYP3A4 (Rif), CYP2C9 (Rif) and CYP1A2 (BNF) and despite the possibility of positive or negative synergies due to the use of both inducers, there is a significant induction in the 3 CYP450 isozymes; such co-administration studies constitute a unique tool to study long-term drug-drug interactions with easy access to the hepatocytes for cell-based assays. In fact, the automated perfusion, as well as the oxygen and pH control in these bioreactors, can be used to expose primary cultures of hepatocyte spheroids to repeated drug dosing, as herein described, or long term time-varying drug concentrations for chronic toxicity assessment.

The maintenance of these hepatic activities and gene expression is enabled by the cell-cell interactions which, during the bioreactor culture, evolve to a liver-like phenotype, as shown by the presence of Albumin, Cytokeratin 18, CYP450 3A and HNF4α (**Fig.s 4.6 a-c**), which are typical hepatic markers. The structural and functional polarity shown by both actin and aPKC staining and CDFDA metabolization (followed by the MRP2-mediated transport through the apical membrane of the hepatocytes) demonstrate that these human hepatocytes cultured as spheroids resume the cuboidal geometry, without actin stress fibers, and a polarized liver-like architecture. The presence of a functional bile canaliculi network in the hepatocyte spheroids, which had not been shown to be functional in previous studies (Dvir-Ginzberg *et al.* 2004), ensures an efficient polarized transport of the metabolic by-products in these hepatic microtissues. The similarity to *in vivo* liver tissue makes this system an interesting tool for fundamental studies of the hepatic functions in physiological or bioreactor-simulated pathological conditions, namely for more complex drug transport studies. Further improvements to this system may be achieved by adding cell-matrix interactions using spheroids encapsulated in alginate (Tostoes *et al.* 2011) or heterotypical cell-interactions, by co-culturing hepatocytes with endothelial cells (Kidambi *et al.* 2009) or fibroblasts (Khetani and Bhatia 2008;

Leite *et al.* 2011). In fact, the preliminary studies on co-culture had shown a stable and effective improved on hepatic functions for the tested culture time, which makes this an attractive strategy to be further explored and adapted to the system for culture human hepatocytes developed in this work.

In conclusion, the perfusion bioreactor system presented herein allows to culture defined size human hepatocyte spheroids (80 μm) which maintain hepatic liver-specific protein synthesis, CYP450, phase II and III drug metabolizing enzymes' gene expression and activity, as well as liver-like architecture inside the spheroids for 2-4 weeks.

5. ACKNOWLEDGEMENTS:

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V

HepaRG IN 3D

This chapter is based on the following manuscript:

3D HepaRG Model
for Toxicokinetic and Toxicodynamic Purposes
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Sofia B. Leite designed, performed and analysed the results of cell culture experiments described in this Chapter. The remaining authors of this work had collaborated in different stages of the study

ABSTRACT

The maintenance of differentiated hepatocyte phenotype and its specific physiological properties is known to depend on several factors, such as chemical signals, cell-cell and extracellular matrix molecular interactions, as well as the use of three-dimensional matrices. The culture of HepaRG as 3D structures in stirred tank bioreactor could represent an added value as an hepatic test system for toxicokinetic and toxicodynamic studies. The use of a cost-effective commercially availability bioreactor for routine use and compatible with high-throughput cell analysis constitutes and attractive approach on the Drug Testing Industry.

In order to address the biotransformation capacity of the bioreactor-based HepaRG system, CYP1A2, 2B6, 2C9 and 3A4 induction, with prototypic inducers, and the activity of the phase II enzyme, UGT were tested. The functionality of the system was demonstrated by albumin secretion capacity, CYP3A4 induction and UGT activities, which have shown stable profiles up to 7 weeks of culture. Immunofluorescence-based measurements demonstrated polarity of transporter expression and formation of tissue-like arrangement including bile canaliculi-like structures. Moreover, bioactivation of acetaminophen (APAP) and its related cytotoxicity was accessed in a system amenable to high-throughput screening and the 3D structures results were compared with platted cells (2D) by means of a cell-based cytotoxicity assay model. The results show the advantage of integration *in silico* models for a better understanding of the *in vitro* cell behaviour. The developed approach had also proved to be a good strategy to reduce the time to obtain fully differentiated cell cultures.

In conclusion, 3D HepaRG cultures performed in stirred tank bioreactors are an attractive tool for toxicological studies, since it showing a liver-like performance and a practical applicability.

CONTENTS

1. INTRODUCTION

It has become increasingly clear that the kinetic and metabolic fate of a compound has an important influence on its toxic potential, disposition in the body and eventual excretion (Coecke *et al.* 2006). Although *in vitro* and *in silico* human metabolic competent test systems are considered essential parts of integrated test strategies for systemic toxicities in general, metabolism is still deemed a bottleneck in *in vitro* toxicological test development. Therefore, in order to predictably screen the toxic compounds, it is essential to develop reliable and relevant human-based *in vitro* test systems that are metabolically competent and will model the hepatocytes process of biotransformation. The *in vitro* results obtained from such a system can be then further integrated by *in silico* computer models (such as physiologically-based toxicokinetic (PBTK) models) and converted into dose-response information for the entire organism enabling to assess the safety profile of compounds. This opens the possibility of using *in silico* strategies on kinetic data to bridge the *in vitro* and *in vivo* paradigm. However, more sophisticated *in vitro* models that maintain the liver function over a long time course for assessing drug metabolism and toxicity are currently needed to improve the predictive power of this approach.

To prove the metabolic competence of any metabolic competent *in vitro* system such as cell lines, liver slices, primary cells or stem cells, the presence and activity of phase I and phase II biotransformation enzymes should be evaluated (Coecke *et al.* 2006). Furthermore, it is important that the metabolic machinery demonstrates to be functional and the polar arrangement of the hepatocytes *in vivo*, with apical and biliary sides characterised by transporters distribution, is present.

Human hepatocyte cultures are considered to be the gold standard for testing liver toxicity since they better reflect what happens in the human liver. However, these cells are rather difficult to obtain and besides undergoing to spontaneously dedifferentiation, their profile is highly dependent on the donor and thus presents a high inter-donor variability. Moreover, they can keep their functionality in culture for a relatively short time.

A good alternative to human hepatocytes is represented by the HepaRG cells developed by Gripon et. al (Gripon *et al.* 2002). This is a human hepatoma cell line, derived from hepatocellular carcinoma that had shown liver-specific functions comparable to the human hepatocytes (Lubberstedt *et al.* 2011). After treatment with 2% (v/v) DMSO, the cells fully differentiate in a co-culture of biliar and hepatocyte-like cells expressing major cytochrome P450 (CYP450) enzymes, nuclear receptors, transporter proteins and transcription factors (Aninat *et al.* 2006; Kanebratt and Andersson 2008). These liver-specific functions can be maintained at high levels in for longer when compared to primary hepatic cultures. Since liver is a 3D organ, in which cells are maintaining cell-cell contacts important for their function and specific polarity, a lot of effort is undertaken to mimic the same conditions *in vitro*. It was already proven that human hepatocytes cultured in stirred tank bioreactors (spinner vessels) as 3D cultures produce tissue-like structures and maintain the liver-specific functions for longer and at higher level (Miranda *et al.* 2009; Leite *et al.* 2011; Tostões 2011; Tostões *et al.* 2011). Since the major application of toxicological models is in expensive screening of compounds, it is an added value that the developed method is compatible with existing standard laboratory equipment and high-throughput techniques (Schutte *et al.* 2011). Moreover, it takes into consideration factors important for toxicological approaches, eg. commercial availability of the system, easiness for routine approach, cost-effectiveness and avoiding the necessity to use matrixes or tubing that could lead to non-specific binding.

The aim of this study was to apply the 3D culture techniques in the stirred tank bioreactor to HepaRG cells, a high standard hepatic cell line. Besides characterization of phase I and II enzymes capabilities, the biocompetency of the developed system was assessed by testing the bioactivation of acetominophen (APAP). This assay also intended to test the applicability of the developed approach in toxicological assays, since APAP was activated to the toxic metabolite. Being one of the major cause of death by liver failure in US (Hawkins *et al.* 2007), APAP is taken as reference hepatotoxic compound for *in vitro* assays.

Experimental data have been analyzed using a process based modelling approach similar to PBTK modelling, but adapted to our cell-based assays with the aim of elucidating the main factors responsible of the *in vitro* behaviour.

Since the HepaRG differentiation is a quite long process (approx. 14 days) and not always complies with the fast drug screening tests, an approach tested previously for neuronal cells was evaluated to decrease the time of differentiation was evaluated (Serra *et al.* 2007).

2. MATERIALS AND METHODS

2.1. HepaRG cell cultures

Cells were provided by Biopredic International (Rennes, France) and maintained as described in Gripon *et al.* 2002 (Gripon *et al.* 2002). Briefly, cells were expanded in 2D flasks for two weeks. Culture medium was composed of William's Medium E (Gibco, Milan, Italy) supplemented with 10% (v/v) of HyClone FetalClone (Celbio, Milan, Italy), 2 mM L-glutamine (Gibco), 1% (v/v) Pen/Strep (Gibco, Milan, Italy), 5 µg/ml bovine insulin (Sigma, Milan, Italy) and 50 µM hydrocortisone (Sigma, Milan, Italy). The 2 weeks process of differentiation was induced by adding 2% (v/v) DMSO (Sigma, Milan, Italy) in the culture medium. Culture medium was changed twice a week. Before CYP induction studies or cytotoxicity testing, cells were cultured in DMSO-free medium for 48h.

2.1.1. Differentiated 3D cultures (3D Diff)

Inoculation of spinner bioreactors was adapted from protocols developed before (Leite *et al.* 2011; Tostões 2011). Briefly, after expansion and full differentiation in 2D monolayers (see 3.1), the cells were trypsinized and $3x10^6$ of cells were inoculated into 125 ml spinner bioreactor (from Wheaton, Techne, NJ) in DMSO-free medium. The cells viability was above 95% as determined by Trypan blue staining. After cell aggregation (at day 3) DMSO was added to the culture up to the final concentration of 2% (v/v) (**Fig. 5.1 A**). Cell spheroid diameter was maintained between 50 and 200 µm by maintaining the stirring rate between 50 and

80 rpm. Culture was maintained for up to 7 weeks, being day 0 the addition of DMSO on 2D culture (**Fig. 5.1 A**).

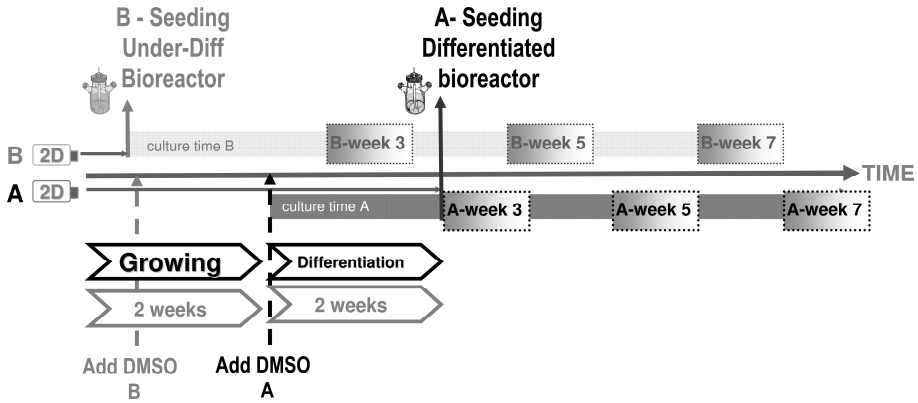

FIGURE 5.1.: Experimental set-up used for 3D HepaRG culture in stirred bioreactor. Cells were inoculated in the bioreactor at two different times: after fully differentiation - Diff (A) and for the differentiation as 3D – U-Diff (B).

2.1.2. Under-Differentiation 3D cultures (3D U-Diff)

After the studies on the characterization of the 3D HepaRG cell culture (3D-Diff), the same strategy was tested to decrease the differentiation time by inoculation the stirred bioreactor earlier in the differentiation process (3D U-Diff) (**Fig. 5.1 B**) adapted from Serra *et al.* (Serra *et al.* 2007). Briefly, the cells were expanded as monolayers in T-flasks and inoculated in a 125-ml spinner bioreactor (from Wheaton, Techne, NJ) at a density of $53x10^5$ cell/ml, before initiation of differentiation process. The cells viability was above 95% as determined by Trypan blue staining. After cell aggregation (day 3), DMSO was added to the culture up to the final concentration of 2% (v/v) (**Fig. 5.1 B**). Cell spheroid diameter was maintained between 50 and 200 µm by maintaining the stirring rate between 50 and 80 rpm. Culture was maintained for 7 weeks starting to count on the addition of DMSO on the bioreactor (**Fig. 5.1 B**).

2.2. Structural arrangement of the cell spheroids

Hepatocyte spheroids were fixed in 4% (w/v) paraformaldehyde (PFA) in PBS for 1 hour at room temperature (RT), blocked overnight (O/N) at 4ºC in 1% (v/v)

Triton X-100 (Sigma-Aldrich) and 0.2% (v/v) fish skin gelatin solution in PBS and subsequently incubated with primary antibodies diluted (1:100) in 0.125% (v/v) fish skin gelatine in PBS for 2 days at 4°C. Cells were washed three times with PBS and secondary antibodies (diluted 1:500 in 0.125% (v/v) fish skin gelatine in PBS) were applied to the cells overnight at 4ºC. After three washes with PBS, the samples were mounted in Prolong gold anti-fade containing 4,6-diamidino-2-phenylindole (DAPI). Cells were visualized using spinning disk (Andor Technology, Belfast, Northern Ireland) confocal microscopy. Primary antibodies used were: goat anti-albumin, mouse anti-P-Glycoprotein (Abcam, Cambridge, UK), mouse anti-Cytokeratin 19 (Santa Cruz, CA. 95060), Alexa 488 conjugated Phalloidin (Molecular 194 Probes, Eugene, OR). As secondary antibodies anti-mouse Alexa Fluor 488 and anti-goat 195 Alexa Fluor 594 (Molecular Probes, Eugene, OR) were used.

2.3. Hepatocyte function assays.

Supernatant samples were taken to quantify the secretion of albumin, synthesis of urea and lactate dehydrogenase (LDH) leakage. Spheroid samples were used to evaluate cell morphology, cell content in the bioreactor and to normalise the values of the measured parameters using Micro BCA Protein Assay kit (Pierce, Italia) (according to manufacture instructions). All samples were analysed in triplicates.

2.3.1 Clinical chemistry indicators

LDH activities were measured using the colorimetric kit Modular P800 (Roche Diagnostics, Mannheim, Germany). Levels of albumin and urea were assessed using the enzyme-linked immunosorbent assay (Albuwell, Exocell, Philadelphia, USA) and the colorimetric kit (QuantiChrom™ Urea Assay Kit, DIUR-500, ref DIUR-500; BioAssay Systems), respectively (according to the manufacture instructions).

2.3.2. Hepatocyte CYP450 enzymes activity.

Cell spheroids were distributed in 96-well plates, approximately $8x10^4$ cell/well, in DMSO-free medium. After 48h in culture, the induction started with the

addition of the reference inducers, namely phenobarbital (PB, 530 µM), rifampicin (RIF, 10 µM) and β-naphtoflavone (BNF, 25 µM). The medium with or without the proper compounds was refreshed every 24h up to 72h. Each condition was repeated in triplicate. At the end of the incubation time, two different procedures were performed for i) screening the major CYP enzymes activity or ii) the determination of long term CYP3A4 induction:

i) Cells were incubated with a selective CYP-substrate cocktail consisting of 26 µM phenacetin (CYP1A1/2), 3 µM midazolam (CYP3A4), 9 µM diclofenac (CYP2C9) and 100 µM bupropion (CYP2B6) (from Sigma). After 30 minutes, supernatants were collected and stored at 20ºC until LC-MS analysis.

ii) CYP3A4 activity was assessed using a luminescence kit (CYP3A4 Luminescent Assay Kit, P450-Glo™, Promega, Italia). The activity refers to the increase of the luminescence from the control (wells with no cells).

In both cases, the obtained values were corrected by the protein content of each well, quantified using the micro BCA kit. Results were expressed as fold increase of the specific CYP activity (pmol/hour/mg of protein or relative luminescent units/hour/mg of protein) when compared to the cells maintained in culture medium with the vehicle (DMSO).

2.3.2.1. LC-MS Analysis.

After cocktail (referred in 3.3.2.i)) incubation, the qualitative and quantitative determination of specific products formed by the respective P450 iso-enzymes, namely acetaminophen (CYP1A1/2), 1´-hydroxymidazolam (CYP3A4), 4´-hydroxydiclofenac (CYP2C9) and hydroxybupropion (CYP2B6) was performed using a liquid chromatography-tandem mass spectrometric method (LC-MS/MS) developed in-house and adapted from (Lubberstedt *et al.* 2011). The internal standard used for quantification was griseofulvin. LC-MS/MS analyses were performed on a Waters Acquity Ultra Performance Liquid Chromatographic system (UPLC) coupled on-line with a Waters Micromass Quattro Ultima Pt triple-quadrupole mass spectrometer equipped with an electrospray ionisation source (Waters, Milford,

MA, USA). Instrument control, data acquisition and data evaluation were performed using the software Waters MassLynx™ version 4.1.

Analyte separation was performed at 40°C using a Waters Acquity UPLC BEH C18 analytical column (1.7 µm, 2.1 x 50 mm) with a Waters Vanguard™ Pre-column (2.1x5 mm) and a crudcatcher. The binary UPLC pump was operated at a flow rate of 400 µl/min. The injection volume was 10 µL and the auto-sampler was operated at 10°C. The eluents used were: (A) 0.1% (v/v) formic acid in water and (b) 0.1% (v/v) formic acid in acetonitrile. The linear gradient used to achieve the separation was as follows: 0-0.4 min: 5%B, 0.4-2.2 min: 5-95%B, 2.2-3 min: 95%, 3.0-4.0: 95-5%B. The retention times of acetaminophen, hydroxybupropion, 4´-hydroxydiclofenac and 1´-hydroxymidazolam were 1.12, 1.46, 1.63, and 1.98 min, respectively.

The separated compounds were detected in positive electrospray ionisation (ESI). The triple-quadrupole mass spectrometer was operated in the Multiple Reaction Monitoring (MRM) mode.

The operating conditions of the mass spectrometer were as follows: capillary voltage, 2.8 kV; source temperature, 150°C;desolvation temperature, 350°; desolvation nitrogen gas flow ,721 L/h; cone nitrogen gas flow, 72 L/h, argon pressure in the collision cell, $3.06e^{-3}$ mbar. The fragmentation parameters were optimised for each of the four analytes and different values of collision energy (eV) were set. The transitions used for quantification were 152.06 > 110.12 (13 eV) for acetaminophen, 255.90 > 238.09 (12 eV) for hydroxybupropion, 342.06 > 324.11 (20 eV) for 1´- hydroxymidazolam and 312.05 > 231.04 for 4´-hydroxydiclofenac (15 eV). For qualification, the transitions used were 152.06 > 93.12 for acetaminophen, 255.90 > 139.13 for hydroxybupropion, 342.06 > 202.98 for 1´- hydroxymidazolam and 312.05 > 266.05 for 4´-hydroxydiclofenac. A dwell time of 0.05 sec was used for all transitions. The lower limit of quantification (LLOQ) was set to 7.8 nmol/L for each analyte.

2.3.3. Uridine diphosphate glucuronoltransferase (UGT) activity.

Uridine diphosphate glucuronoltransferase (UGT) activity was determined by quantifying the remain substrate, 4-methylumbelliferone (4-MU), after incubating with the cells. The UGT activity was measured both in 2D and 3D HepaRG cultures. The procedure was performed according to Gomez-Lechon *et al* (Gomez-Lechon *et al.* 1997) with slight modifications. Briefly, 100 µM solution of 4-MU in 0.01M PBS was incubated with cells for an hour at 37ºC. Fluorescence samples were analysed at an excitation wavelength of 320 nm and emission of 450 nm. At the end of incubation with 4-MU, the protein cell content in each well was assessed as described above to normalise the specific UGT activity. The 4-MU remaining concentration in the supernatant solution was determined based on a standard curve generated in PBS spiked with substrate. Each condition was performed in triplicate. The activity of UGT was expressed as µmol of 4-MU metabolized *per* hour and *per* mg of protein.

2.4. Biocompetency assessment - Acetaminophen (APAP) Toxicity.

Cells spheroids were distributed in 96-well plates, approximately $8x10^4$ cell/well, in DMSO-free medium. The APAP concentrations used to generate a dose response curve were chosen based on previous determination of the EC50 on 2D HepaRG cultures. After 48h incubation at 37ºC, the assay starts by adding, in triplicates, different concentrations of APAP: $6.60x10^3$, $3.30x10^3$, $1.65x10^3$, $8.25x10^2$, $4.13x10^2$, $2.06x10^2$, $1.03x10^2$, 51.6 and 25.8 mM. Culture medium with proper concentrations of compound was refreshed every 24h up to 72h. Cell supernatants were collected every 24h for LDH activity measurement as described before.

2.5. Cell-based assay model

A coupled fate, transport, population growth and toxicity model has been recently developed to analyse cell-based assays. The model has been previously described in Zaldívar *et al.* (Zaldívar *et al.* 2011; Zaldívar *et al.* submitted) where it was implemented for several types of platted cells. If we assume that the differences between both types of culture systems, 2D and 3D, are due to the transport of APAP

to the cells located in the interior of the agglomerate and since diffusion is the sole transport mechanism, it is possible to define an effective diffusion coefficient, D_e, as in transport on porous media (Fogler 1999):

$$D_e = \frac{D \cdot \phi}{\tau}$$

where ϕ is the porosity of the cell aggregate and is the tortuosity factor defined as the actual travelled distance between two points divided by the Euclidean distance. Since this diffusion coefficient is related to the APAP transport to the cell we can assume that there will be a decrease of APAP transport comparing 2D and 3D experiments, and therefore, a decrease in estimated cytotoxicity. To estimate this value, we have used the 2D experiments to adjust the toxicity model's parameters –referred to internal cellular concentrations-, i.e. *NEC* (No Effect Concentration) and k_t (killing rate) , see Koijman and Bedaux (Kooijman and Bedaux 1996) and the transport parameter (uptake rate); and the 3D experiments to calculate the value of the (ϕ / τ) factor. In addition, this has allowed us to analyze, if along the duration of the experiments, there have been changes in the structure of the spheroids.

2.6. Calculations and statistical analysis

Albumin secretion, CYP 450 enzymes activity, UGT activity and APAP cytotoxicity were evaluated. Albumin secretion was expressed as μg of albumin per day of culture per 10^6 cells. CYP induction was expressed as improvement on specific enzymes activity when compared to the medium control. UGT activity was expressed as μmol of substrate (4-MU) disappeared per hour of incubation *per* mg of total cell protein. APAP cytotoxicity was calculated based on the percentage of viable cells in comparison with the untreated control cells.

Data was gathered from two bioreactors with at least three replicates for each tested concentration. Statistically significant differences between the mean values related to all tested endpoints were determined based on analysis of variance (ANOVA single factor analysis, with α=0.05). Values of $p<0.01$ were considered as

statistically significant. All calculations were done using Microsoft Excell's data analysis toolpack (VERSION 2003).

3. RESULTS

3.1 Hepatocytes function assays – clinical chemistry indicators (3Diff)

The amount of cells in culture was determined by total protein content and neither cell growth nor significant cell death was detected during the culture time (data not shown). Stable maintenance of cell content for the 7 weeks was confirmed by the low release of LDH into the culture medium (data not shown). The albumin secretion capacity of the cells decreased over the time from 11.7±3.7 µg albumin/day/10^6 cells on 3rd week to 6.0 ± 2.3 and 3.5 ± 1.4 µg albumin/day/10^6 cells, on the 5th and 7th weeks respectively. No urea production was detected over the whole tested period.

3.2. Structural arrangement of the cell spheroids (3D Diff)

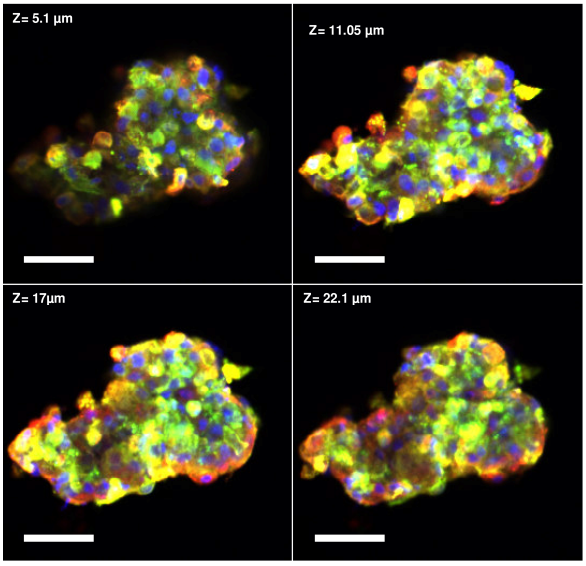

FIGURE 5.2.: Cell arrangement within HepaRG 3D culture. Spheroid showing the two types of cells in culture: hepatocytes (red) and biliary cells (green). Cell nuclei from both cells are stained in blue. White bar corresponds to 50 µm.

Immunofluorescent pictures showing the spheroid characterization are presented in **Figures 5.2** and **5.3**. It can be observed formation of tissue-like

structures. **Figure 5.2** shows the polarization of biliary marker CK19 (green) at the core of the cellular structure, while the hepatocyte-like structures stained for albumin (red) were situated at the outside sphere, as it is visible once the focus move away from the core of the spheroid.

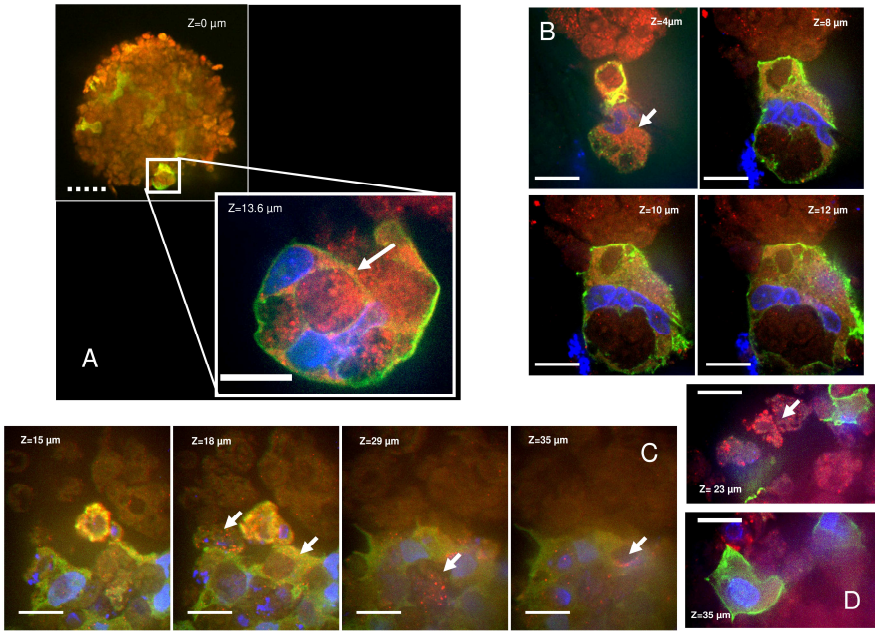

FIGURE 5.3.: 3D organization of the HepaRG spheroids. (A-D) Details of a spheroid showing the polarization of transporter (white arrow). F-actin (green) localizes at the level of cell membranes and it is enriched in bile-canaliculi-like structures. Red stains for PGP protein transporter. White dash bar (A) corresponds to 50 µm; white full bar corresponds to 15 µm.

The same polarized structure is supported by **Figure 5.3** that shows spheroid details where it can be seen that the Pgp efflux transporters (red) are not randomly distributed, but accumulated near the green actin staining, indicating the formation of biliary canaliculum-like structures. The vicinity between the two structures is more visible when moving along the depth of the spheroid (z-axis) where it can be seen the superposition of the biliary structures on the zones where Pgp is accumulated (white arrows), showing an *in vivo*-like cellular arrangement. In both figures, the yellow appearance indicates the superposition of both structures.

3.3. Phase I and Phase II hepatocyte enzyme activities (3D Diff)

3.3.1 Functional activity of CYP450 enzymes

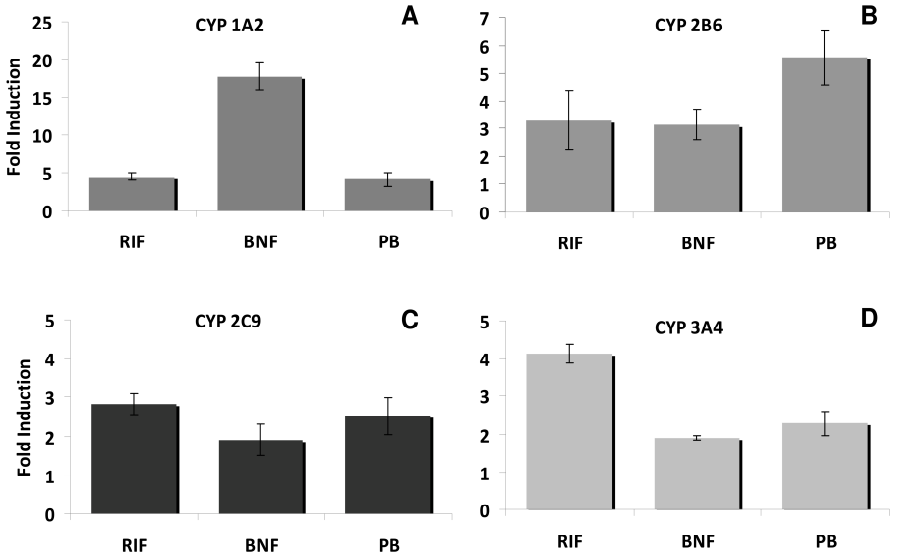

FIGURE 5.4.: Phase I enzymes activity in HepaRG 3D cultures. Induction of CYP1A2 was assessed by 25 µM of β-naphtaflavone (βNF), and CYP2B6 was induced by 500 µM phenobarbital (PB). Induction on both CYP2C9 and CYP3A4 was assessed by adding 10 µM rifampicin (RIF). The assay was performed 3 weeks after the addition of DMSO (used for cell differentiation). Results are mean ± SEM (n =3)

By measuring the appropriate metabolite concentrations by the LC-MS/MS method it is possible to quantify the level of CYP450 enzyme activity (relative to a control). **Figure 5.4** shows the functional activity of the major phase I enzymes in 3D HepaRG culture. After 72h incubation with the respective positive inducers, all tested CYPs showed more than 2-fold induction increase (as recommended by FDA Draft Guideline for Drug-Drug Interactions). Induction rate catalysed by CYP1A2 phenacetin-O-dealkylation was found to be 17.8±1.8. The induction rate for bupropion-hydroxylase, characteristic for CYP2B6 was observed at the level of 5.6±1.0. Both, CYP2C9 and CYP3A4 were effectively induced by RIF as the induction rates of midazolam-1'hydroxylation and diclofenac-4'-hydroxylation were found to be 2.8±0.3 and 4.1±0.3, respectively.

3.3.2 Long term characterization of biotransformation- determination of CYP3A4 and UGT activity

The most representative enzymes of the biotransformation phase I and phase II were selected and their long term biotransformation capacity was assessed by repeated assays on the 3rd, 5th and 7th weeks (**Fig. 5.5** - black bars). The CYP3A4 enzyme functionality is presented as fold induction after addition of the specific indicated compounds (**Fig. 5.5 A**). Stable induction of CYP3A4 was shown during the whole culture period (up to 7th week). The induction rate of more then 2-fold was observed when the cells were exposed to RIF and PB. The values of 7.15±1.84 and 2.76±0.29 were obtained, respectively. No induction was observed upon exposure to BNF.

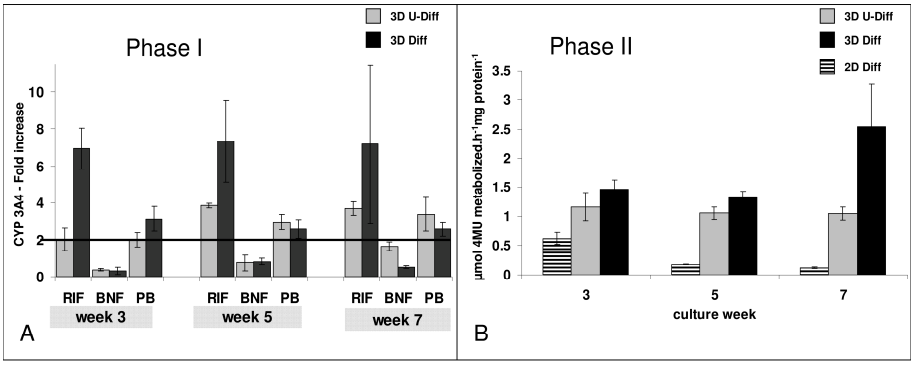

FIGURE 5.5.: Long term biotransformation capacity of HepaRG cells cultured as 3D structures in stirred bioreactor. Results are expressed as mean values of two independent culture runs, with 3 test replicates for each testing condition and error bars correspond to SEM.
A - Phase I enzyme activity represented by CYP3A4 induction after addition of reference inducers. Two different approaches in terms of cell culture are represented (light grey bars represent cultures seeded before the differentiation phase (3D U-Diff) and dark grey bars represent those seeded at the differentiated stage) Black horizontal line indicates the threshold value, from which it is considered that the enzyme activity has been induced.
B- Phase II enzyme activity - represented by UGT. Two different approaches in terms of cell culture are represented (light grey bars represent cultures seeded before the differentiation phase (3D U-Diff) and dark grey bars represent those seeded at the differentiated stage). The results are compared to the 2D cultures (striped bars).

For the first time, accordingly to our knowledge, the activity of the phase II enzyme UGT was measured in 2D and 3D differentiated cultures (**Fig. 5.5 B-** stripes and full black bars respectively). The UGT activity was detected by the

disappearance of the enzyme main substrate 4-methylumbeliferone. In both, 2D and 3D cultures, the UGT activity was observed over the whole period tested. The UGT activity in 2D cultures decreased over the time, whereas in the 3D cultures it was maintained at the stable level from the 3rd to the 5th week and increased on 7th week. Moreover, the 3D model increases the UGT activity when compared to the 2D cultures by approximately 2-20 times.

3.4. Biocompetency: APAP toxicity (3D Diff)

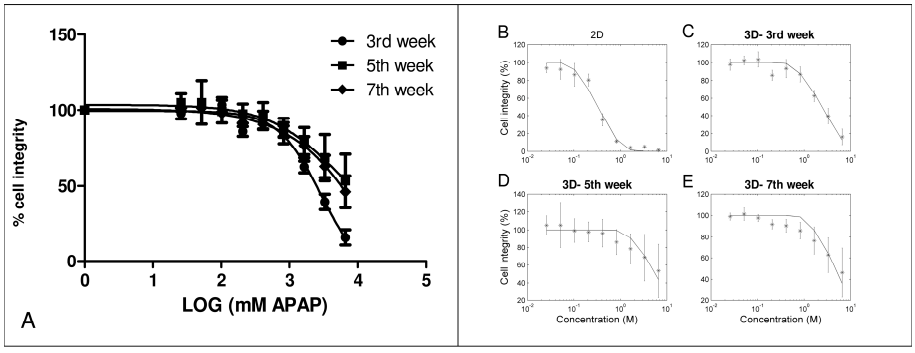

FIGURE 5.6 Bioactivation capacity of the cells. A – Concentration - dependent APAP toxicity at different culture times. Each point corresponds to the average value of triplicate determination of cell integrity, at 24, 48 and 72h of incubation from four stirred bioreactor cultures. B-E – Experimental and simulated dose response curves after addition of APAP at different concentrations at different culture times. B curve, corresponds to the monolayer HepaRG cells (2D) where the other curves show the comparison between 3D experiments and simulations for 3rd, 5th, and 7th weeks assuming a change in the effective diffusion of APAP.

To check the applicability of our 3D developed model in toxicology, the cells were exposed to different concentrations of acetaminophen for 72h. Since the APAP itself is not toxic but has to be metabolized first to its active metabolite – NAPQI, the performed study is also a way to test cells bioactivation/biocompetency capacity. The results are showed on **Figure 5.6 A.** Among the 3 tested weeks (3rd, 5th and 7th), the profiles are similar and concentration- dependent decrease of cell viability is observed. However, on the 3rd week higher toxicity of APAP at the same range of concentrations is observed.

3.4.1. Simulated results

Figure 5.6 B shows the experimental and simulated results for the 2D experiment that was used to fit model parameters as well as the obtained results for 3, 5 and 7 weeks of the 3D culture (**Figs. 5.6 C-E**). The fit of the model parameters in 2D (**Fig. 5.6B**) followed the procedure described in Zaldívar *et al* (Zaldívar *et al.* submitted). For the fit of the 3D culture, the same parameters were used, but applying the factor (ϕ / τ) to the effective diffusion and hence to the APAP transport to the cells. The calculated values for the decrease in mass transfer (mol l^{-1} s^{-1}) related to the platted cells are: 1581, 3844, and 3199, respectively.

3.5. Decreasing differentiation time in 3D (3D U-Diff)

After characterizing the 3D HepaRG cultures in stirred systems, it was tested if, using the same system, the differentiation time of HepaRG could decrease by seeding the bioreactor earlier, i.e. when the necessary amount of cells after expansion was reached (**Fig. 5.1B**), (**3D U-Diff**). CYP3A4 induction and UGT activity were assessed as main endpoints (**Fig. 5.5**, grey bars) and were compared with 3D Diff culture. Regarding CYP3A4 induction (**Fig. 5.5A**), the positive inducers (RIF and PB) acted as such and induced the enzyme to the same extent. No higher induction capacity for RIF was observed (as it was the case in the 3D Diff cultures). There was no significant difference regarding PB induction between the two 3D cultures and no time-dependency was observed. RIF induction potential was much lower in 3D U-Diff then in 3D Diff and stability was reached later (from the 5th to the 7th week).

Regarding the UGT activity, the 3D U-Diff culture has stable activity for the whole period (**Fig. 5.5B**). The values are close to the ones of 3D Diff cells, apart from the 7th week, where 3D Diff activity is much higher. For the whole tested period, the UGT activity seems to be higher then those in 2D culture.

However, further studies are necessary in order to understand the impact of decrease the differentiation time of HepaRG.

4. DISCUSSION

Central role of metabolism in mediating liver response to drug become one of the crucial aspects, addressed in pharmaceutical drug discovery and development. Different strategies have been envisaged setting up *in vitro* systems to prolong cell survival, maintain liver-specific functions and mimic *in vivo* like environment. Various approaches combining co-culture, 3D organization and perfused flow have been developed (Leite *et al.* 2011; Schutte *et al.* 2011; Tostões *et al.* 2011; Zeilinger *et al.* 2011). Several studies have shown that 3D cell-cell contacts reduce the gap between cell culture and real tissue (Pampaloni *et al.* 2009). The liver-like arrangement together with the stratified organization, makes 3D model closer to the *in vivo*, with the ability to give more reliable results (Dhiman *et al.* 2005; Tung *et al.* 2010).

In parallel, new cell sources are explored as an alternative to primary human hepatocytes. One of the most attractive alternative in terms of long-term maintenance of liver-specific competency are the HepaRG cells (Aninat *et al.* 2006; Guillouzo *et al.* 2007; Turpeinen *et al.* 2009; Andersson 2010). Besides showing metabolic profiles similar to human hepatocytes (Lubberstedt *et al.* 2011; Zanelli *et al.* 2011), recently it was also shown that HepaRG have a genetic similarity of 81 to 92% with human hepatocytes (Rogue *et al.* 2011). However, it still shows some limitations regarding the application of *in vitro* testing (Schulze *et al.* 2011) and 3D models to culture HepaRG in an architecture that better resembles the liver itself are still scarce (Darnell *et al.* 2011; Hoekstra *et al.* 2011).

In this work we established a strategy to culture functional 3D HepaRG spheroids in stirred tank bioreactor up to 7 weeks. Mimicking the *in vivo* liver morphology, the cell spheroids were composed of both, biliary-like and hepatocyte-like cells, showing a polarized cell arrangement also supported by polarization of the transporters from the MRP family. This type of cellular arrangement was already shown in the HepaRG 3D cultures (Darnell *et al.* 2011) and human hepatocytes 3D spheroids cultured in stirred systems (Tostões 2011). Polarized expression of transporters in cultured hepatocytes is of outmost importance for *in vitro* drug-drug

interaction studies (Nakanishi *et al.* 2011), as well as test studies such as transfection with hepatitis B virus (Schulze *et al.* 2011).

The stable functionality of our bioreactor-based hepatic system over the tested period was demonstrated by several endpoints. The capability of the system to secrete albumin remains active for 7 weeks, even if decreasing with time. Moreover, comparing the results (11.7 ± 3.7 µg albumin/day/10^6cells at the 3rd week) to those obtained in 2D HepaRG cultures (5 µg albumin/day/10^6cells cells) (Lubberstedt *et al.* 2011) for the same period of time, an improvement of the albumin secretion capacity was shown. Like in the study of Lubberstedt at al. (Lubberstedt *et al.* 2011), no urea synthesis competence of HepaRG cells was observed in our system. This may be due to the fact that in HepaRG cells, ammonia is predominantly fixed into glutamine rather that converted to urea, as previously described by Hoekstra (Hoekstra *et al.* 2011).

We also observed that the established 3D HepaRG cell cultures in the stirred bioreactor maintain the functional metabolic machinery of the main phase I CYP P450 biotransformation enzymes, namely CYP1A2, CYP2B6, CYP2C9 and CYP3A4. All iso-enzymes were present in the system showing diverse basal activity. About 10 times lower activity was observed for CYP1A2 and CYP2B6 when compared to CYP2C9 and CYP3A4 activity (data not shown). As expected (Westerink and Schoonen 2007; Abadie-Viollon *et al.* 2010), the tested iso-enzymes were specifically induced by their respective reference inducers (BNF for CYP1A2, PB for CYP2B6 and RIF for CYP2C9 and CYP3A4, respectively). The induction levels in case of CYP1A2 and CYP2C9 (17.8±1.8 and 2.8±0.3 respectively) are in agreement with the values obtained for HepaRG cultured 2D conditions (Turpeinen *et al.* 2009; Lubberstedt *et al.* 2011). Higher induction was observed for CYP2B6 and 3A4, being closer to the values seen in human hepatocytes (Lambert *et al.* 2009; Turpeinen *et al.* 2009).

Among the human CYP P450 enzymes, CYP3A4 monooxygenase plays the major role in the biotransformation, catalyzing the metabolism of about 60% of drugs (Lehmann *et al.* 1998). Therefore the long term biotransformation capacity of our test

system was evaluated based on the activity of this enzyme. CYP3A4 was strongly induced by RIF up to the 7th week of culture. The 2.5±0.5 to 7.3±2.2 fold-induction was observed. Knowing that the effect of RIF on CYP3A4 varies considerably in both *in vivo* and *in vitro* human hepatocytes, our results indicate that in the established stirred 3D system the induction capacity of CYP3A4 is relatively stable. In previous studies the variation from 1.4- to 7.4-fold was documented in *in vivo* conditions (Floyd *et al.* 2003; Yu *et al.* 2004; Abadie-Viollon *et al.* 2010), whereas the range of 2- to 19-fold induction was shown *in vitro* (Abadie-Viollon *et al.* 2010; Tostões 2011). Regarding HepaRG cultures, published results vary between 2- to 11-fold (Kanebratt and Andersson 2008; Darnell *et al.* 2011). The lower response of CYP3A4 expressed in 3D HepaRG culture to the alternative inducer – PB, is also in agreement with previous studies in both, 2D human hepatocytes and HepaRG cells (Turpeinen *et al.* 2009; Richert *et al.* 2010).

To assess the overall metabolic competency of our 3D *in vitro* system, we also checked the activity of the biotransformation phase II enzyme – UGT, which catalyses more then 35% of the conjugation reactions in human drug metabolism (Trubetskoy *et al.* 2007). According to our knowledge, no previous published data referring to UGT activity in HepaRG cells are available. Therefore, we tested it in both 3D and 2D cultures. 3D cultures showed improvement in the UGT activity from 1.9- to 21-fold, when compared to 2D. Similar findings were made with rat hepatocytes which, when cultured in the same system, increased the UGT activity up to 6 times (Miranda *et al.* 2009).

The practical applicability of the developed system for toxicological studies was evaluated by assessing the mode of action of acetaminophen (APAP) - a known liver toxicant. APAP dose-dependent toxicity was observed, proving the capability of our system to accurately mimic the mechanisms of APAP toxicity seen *in vivo*. In general, to express the toxicity, the formation of a reactive APAP metabolite (NAPQI) is necessary, which is followed by glutathione depletion, mitochondrial dysfunction and ultimately cell death. The results obtained in our study provided evidence of the concerted action of CYP 2E1,CYP3A4,CYP1A2 enzymes and the MRP2 and MRP3

transporters, involved in the described toxic path, up to 7 weeks. The fact that the curves are similar among the tested 72h at different time points (3rd, 5th and 7th week), shows that the daily death rate of APAP is constant, which is in agreement with *in vivo* toxic action of the compound, since it causes acute liver failure and not chronic toxicity (Kaplowitz 2005). However, the highest toxicity of APAP was detected only on the 3rd week at concentrations higher then 1650mM, which could possibly indicate higher activity of the complex MRP-CYP.

The observed APAP non-toxic concentrations are not in agreement with the available *in vivo* data (Gibb and Anderson 2008), however, an *in vitro* model itself can not generate the exact human toxic concentrations. According to Pelkonen et al (Pelkonen *et al.* 2008), there are still several *in vivo* phenomena regarding ADME (Absorption, Distribution, Metabolization and Excretion) process which can not be mimicked in simple *in vitro* models. This might be even not necessary if a stable correlation between the obtained *in vitro* values and the known *in vivo* effects is identified and serves to develop an extrapolation models such as physiologically based toxicokinetic (PBTK) models (Pelkonen *et al.* 2008). In this work, we have used a cell-based assay model to estimate the *in silico* reproducibility of the *in vitro* results and the differences between 2D and 3D assays as a first step towards a strategy for future *in vitro-in vivo* extrapolation (IVIVE). The used cell-based assay model was able to generate the experimental profiles considering different diffusion parameters based on the porosity/tortuosity of the 3D spheroid. The values obtained for the decrease of the mass transfer for weeks 3, 5 and 7 show that the differences observed on APAP toxicity might not be fully related with the metabolic machinery, but with the different exposure of the cells to the compound. The results suggest that, on 3rd week, the cells are exposed to the compound in a manner which resembles 2D model, loosing this feature over the following weeks. This could be caused by stronger cell-cell connections, expressed after long time in culture. However, further studies need to be performed to understand the reasons and the mechanisms of the observed diversity. The actual study intends to demonstrate the applicability of the 3D model in toxicity studies, and the further advantage of the integration of *in silico*

models, towards the extrapolation and understanding of the data, being the first time that this was performed in a HepaRG 3D system.

After having an extended characterization of the 3D HepaRG system, we tried to decrease the differentiation time of HepaRG as it was previously done with neuronal cells (Serra *et al.* 2007). The 3D U-Diff culture was characterised in terms of biotransformation capacity and compared with the 3D Diff culture. As it happened with 3D Diff cells, CYP3A4 positive and negative inducers had worked as such also in the 3D U-Diff culture. However, RIF induction potential in 3D U-Diff was lower then in 3D-Diff. This might mean that the CYP3A4 was still not mature enough. From the other hand, this diversity might also reflect the observed *in vivo* hepatic variability upon exposure to RIF, which was mentioned before. Regarding UGT activity, 3D U-Diff has a profile very similar to the 3D Diff, with the same improvement from the 2D cultures and similar activities on weeks 3 and 5. However, on the 7th week, instead of expected increase, the UGT activity remained constant. There is a probability that this improvement would be observed latter on.

In spite of observing some lower biotransformation activities, the 3D U-Diff is still a valid model for 3D hepatic cultures since it reaches the minimal conditions such as responding to induction and having a stable metabolic profile for 7 weeks. The results lead to believe that the earlier aggregation of HepaRG helps in the early maturation of biotransformation enzymes. Furthermore, the applicability of the 3D HepaRG U-Diff model should be considered depending on the needs: from one hand site, having Diff and U-Diff cultures can mimic the natural *in vivo* variability, from the other hand site, instead of being applied in toxicokinetic (biotransformation) U-Diff can function as quicker source of cells for application in toxicodynamic studies (mechanisms) since the cell seem to be maturated.

Although other 3D HepaRG bioreactor studies had already been performed with successful results (Darnell *et al.* 2011), our system has the advantage of lacking structures that would lead to non-specific bindings and the possibility of on-line cell sampling, allowing the cell characterization and quantification during the culture time. Additionally, the easy access to the cell content with minimal perturbation of the bulk

culture permits the bioreactor to work as feeder model, generating big amount of cells in the same conditions that can be further distributed through smaller high-throughput models for screening of compounds. Moreover, stirred spinner bioreactor is a simple and inexpensive bioreactor system (Rauh *et al.* 2011), that can be easily up-scaled (Leite *et al.* 2011) for higher amounts of cells for feeding.

In summary, the 3D HepaRG cultures in stirred tanks are a good model to maintain hepatocyte-like features, showing applicability for toxicological studies and integration with *in silico* models. According to our knowledge, this is the first time that such approach was tested in HepaRG 3D system. Moreover, it was shown that the system can generate early differentiated hepatocytes mimicking natural variability *in vivo*. With further improvements and good extrapolation models, this is a promising and attractive strategy for drug testing applications for both, pharma industry and CRO's.

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DISCUSSION AND CONCLUSIONS

Sofia B. Leite has written the whole chapter, based on the referred papers and her own results described in chapters II to V.

CONTENTS

1. DISCUSSION

The development of *in vitro* methods to culture metabolic competent hepatocytes can have different applications such as the generation of cells for liver engraphment or bioartificial liver (BAL), drug testing, toxicokinetic studies or even for studying liver paths and responses following a more toxicodynamical assessment under physiological and pathological conditions. An overall improvement of the hepatic culture system is needed such that it reacts as close as possible to the *in vivo* cells. However, depending on the application, there are some parameters that gain or loose relevance for the optimization of the method.

In this work the 3D spheroid strategy for hepatocyte culture in stirred tank vessels directed at drug testing was pursued as a way to predict toxicity, bioactivation and drug-drug interaction, with a special focus on applications for the Pharma Industry. Within this context, *in vitro* methods for these applications should take into account specific characteristics, as listed in **Table 6.1**.

Table 6.1.: Aimed characteristics of an *in vitro* method for drug testing applications in the Pharma Industry

Cells	➢ generation of metabolically competent cells ➢ metabolic profile similar to *in vivo* ➢ time-stable metabolism
Tests	➢ acute toxicity ➢ repeated dose effects ➢ chronic toxicity ➢ drug-drug interactions
Drug Testing	➢ low amounts of compounds ➢ screening ➢ compatible with high-throughput ➢ low drug interaction apparatus and scaffolds
Transferability	➢ easy to work ➢ easy cell access ➢ reproducibility ➢ robustness ➢ scalability
Costs	➢ quick generation of cells ➢ time effective strategies (large numbers of results obtained in a short period of time) ➢ cost-effective apparatus

Having these factors in mind, our strategy was developed by using three different cell types and distinct approaches. Each study conquered novel outcomes that constituted the base line for the next study or disclosed additional knowledge about the system. The main outcomes obtained in each step are summarized in **Figure 6.1** as an update from the aims summarized in **Figure 1.8**.

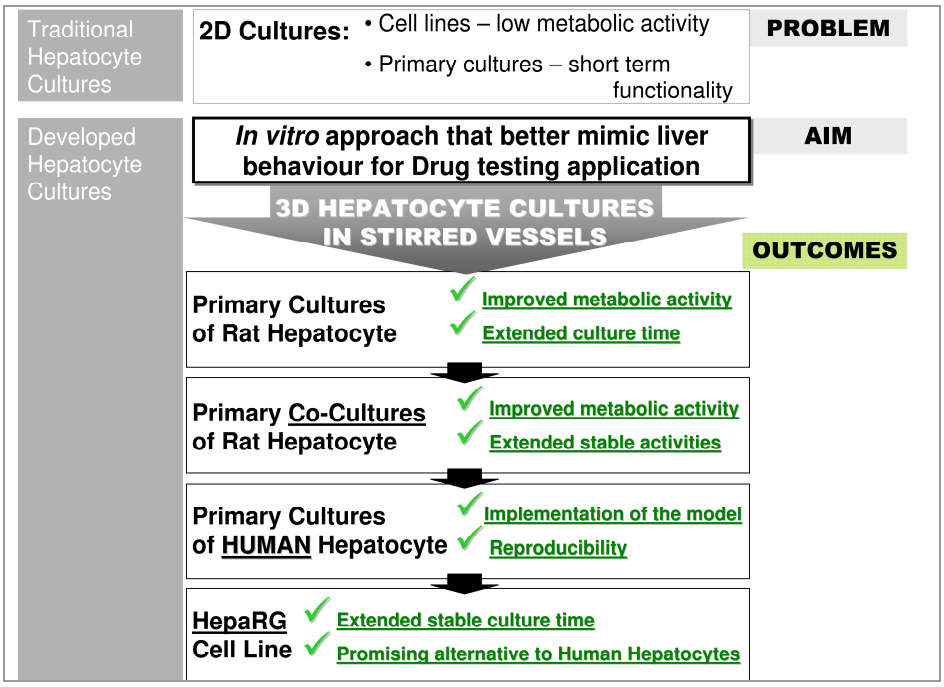

Figure 6.1: Main outcomes from the studies performed in this thesis. This figure is based on the aims listed in figure 1.8 (*Chapter I - Introduction*)

1.1. The cellular models to predict liver function

As discussed in detail in **Chapter I**, animal models, although being integrative models, have several limitations for test studies. On one hand, the test in an animal as a whole does not allow the assessment of the cell and organ reactions in detail, making it impossible to understand the mechanisms of toxicity. On the other hand, the minimization of the impact of inter-species variability between humans and animals has lead in the past to several mistakes such as the use of Thalidomide (Nau 1986). Thus, the relevance of having human-derived *in vitro* models that can generate reliable data regarding the pharmacokinetics of ADME, especially on metabolism, is obvious.

For many years, microsomes have been a powerful *in vitro* tool to understand biotransformation. However, in such simplistic system, it was impossible to express the whole cellular metabolic equipment; therefore, cell cultures started to be the most obvious option.

The present work aimed at developing a model that could generate highly competent human liver cells. Since freshly isolated human hepatocytes are not easily obtained as a regular source and, when in culture, their viability and functionality is often compromised (Schutte et al. 2011), our first approach in this work was to use rat hepatocytes (**Chapters II** and **III**). In addition to being cells with high metabolic activity, leading usually to results reproducible in human cells, the generation of a more robust and reliable cell culture models of rat hepatocytes is also a way to accomplish the 3R's. Having *in vitro* models that can predict animal toxicity by sacrificing less individuals will further reduce and refine animal testing in the pre-clinical phase.

Finally, **Chapter V** describes the development of a culture strategy using a human cell line as a way to overcome the limitations of primary cultures of human hepatocytes.

Having a cell line that can perform similarly to human hepatocytes would be valuable both for practical and economic reasons, already stated in **subsection 3.3**

of **Chapter I**. HepaRG has been presented as one of the best alternative candidates; however, there are already other cell lines under development with promising hepatic profiles such as the case of FLC cells (Laurent *et al.* 2011). Moreover, hepatocyte-like cells derived from stem cells are also a relevant and a very promising approach to generate high amounts of hepatocytes. Additionally, in spite of not being addressed in this work, remarkable results towards the hepatic stem differentiation have been achieved by using 3D strategies and bioreactors (Laurent *et al.* 2011; Miki *et al.* 2011).

The use of 3D models in stirred bioreactors has been shown to improve the differentiation of under-differentiated cells with other cell types (Serra *et al.* 2007), as well as here when using HepaRG cells (**Chapter V**). Based on these studies it is expected that stirred bioreactors would represent a good strategy to differentiate and characterize hepatocyte-like cells.

1.2 The accomplishment of metabolically competent cultures

Having physiological-like liver cultures consists in maintaining hepatocytes that can perform as the liver and mimicking all its functions. However, when the study aims to assess toxic effects, biotransformation of drugs has a more relevant role. The work of this thesis, besides tackling secretion functions (such as albumin and urea) focuses on biotransformation enzymes, their activities improvement and maintenance. Moreover, in addition to assessing the basal activity of the developed culture model, it has also been challenged to respond to reference compounds that affect phase I enzymes by performing studies of Induction, Clearance and Bioactivation. In each Chapter, the strategies employed to monitor the cultures were not exactly the same in order for the culture model to be more extensively characterized. Thus, the best way to analyze the different studies transversely is by comparing their activity induction hills, resumed on **Table 6.2**.

Table 6.2.: Resume of CYP activity induction obtained in this thesis

			Fold-induction
CYP 1A/2B	Rat hepatocytes in Co-Culture (***Chapter III***)	5% pO_2	1 – 1,5 x
		30% pO_2	2 – 3 x
		70% pO_2	1 – 1,6 x
	Human hepatocytes (***Chapter IV***)	Donor A	1 – 2,6 x
		Donor B	2 – 3 x
		Donor C	15 – 19 x
	HepaRG (***Chapter V***)	Diff	5 – 20 x
CYP 3A4		Under-Diff	2 – 4 x
		Diff	3 – 10 x

Besides the difference on the assessed CYP's and the inter-donor variability it can be observed that it was possible to obtain the same range of fold-induction in the different studies, in agreement with *in vivo* data (Floyd *et al.* 2003; Gorski *et al.* 2004; Kharasch *et al.* 2004; Yu *et al.* 2004; Abadie-Viollon *et al.* 2010). In general terms, the strategy developed with rat cells was also applicable to human cells. Moreover, the values and the variability observed for the HepaRG cells are within the range detected for freshly isolated hepatocytes.

Drug-induced liver injury (DILI) represents a major challenge for clinicians, the pharmaceutical industry and regulatory agencies worldwide (Kaplowitz 2005). DILI effects, in particular idiosyncratic reactions highly depend on individual specific responses to xenobiotic biotransformation. It is then important that the hepatic cellular models, at the same time that they improve metabolic profile can also reflect its natural *in vivo* variability. The work presented on **Chapters IV** and **V** has shown that besides being a robust and reproducible approach, the 3D mode to culture cells using stirred tanks can however preserve or generate human CYP variability.

Nevertheless, extrapolating *in vitro* values to *in vivo* is limited. For instance, considering the different compound partitioning in both situations, *in vitro* and *in vivo,* the real concentration at which cells and the organ are exposed varies. The concentration of the compounds in the plasma is affected by protein binding and hydrophobic compartments like phospholipid bilayers or hydrophobic proteins and thus it is very difficult to predict the compound concentration at which the organ is exposed (Pelkonen *et al.* 2008; Zaldívar *et al.* submitted).

As mentioned before, the accomplishment of a predictive strategy of human toxic values will probably not be possible only with metabolic *in vitro* models.

A recent strategy to obtain a better prediction of human risk is to use the so called humanized models, where human cells are transplanted into rat or mice, having a human metabolization (Chen *et al.* 2011). However this strategy needs to use the cells generated *in vitro* and works on only 1R (Refinement) of the 3R's policy.

Recently, human microdosing is becoming a prospective strategy to better understand human metabolism and potentially avoid the use of animals. It consists of testing the compounds already tested *in vitro*, in human volunteers using very small doses in order to avoid toxicity, but allowing to trace the resulting metabolites (Ings 2010), but still the tests are based on *in vitro* outcomes.

In any case, the *in vitro* metabolic models need to generate data that can be correlated directly with the *in vivo* outcome and some 3D models have already shown to be able to perform such correlation (Toh *et al.* 2009).

1.3. The 3D approach as an answer to the need for alternative methods

The developed work confirmed that culturing hepatocytes in a 3D arrangement rather than on the typical 2D monolayers on top of a flat and rigid surfaces, can improve their specific functionalities and be maintained for longer periods. The adopted approach consisted of maintaining the cells as spheroids, where the cells could have an elevated number of cell-cell contact without using any scaffold. We

intended to restore the tissue-specific architecture, biochemical cues and avoid the loss of cell–cell communication while at the same time reducing the gap between cell culture and physiological liver.

As result of toxicity, *in vitro* as well as *in vivo* cell death, can be due to either apoptosis or necrosis; different body reactions characterize these two paths. These reactions still cannot be predicted by the simple *in vitro* systems, and many times not even the type of induced cell death is in agreement with what happens *in vivo* (Kaplowitz 2005).

A strategy to better mimic the several occurrences and reactions taking place in the body is to use integrative and multiparametric approaches such as co-culture. The inclusion of heterotypic cell-cell interactions in the 3D models and the reciprocal effect of different cell populations on the whole microenvironment should hence be carefully considered when trying to establish physiologically relevant *in vitro* models of tissues/organs. In this work, co-culture was a transversal strategy used in two types of approaches: as feeder cells, as for the fibroblasts (**Chapters III** and **IV**) or as helper cells, as for biliary cells (**Chapter V**) that help on hepatic metabolism by being involved in the excretion and generating polarization.

For co-culture, it is relevant to use cells that mimic the liver milieu; however, none of these cells should interfere with the hepatic metabolism but allow for hepatic metabolites to diffuse to the target cells, without further biotransformation. Including cells, with specific zonated distribution that will aid hepatocytes on the excretion of the metabolites, such as biliary cells, as observed in HepaRG co-cultures, will make the model more realistic. On the other hand, when the co-culture cells work uniquely as feeders, instead of polarized distribution, it is intended to have an homogeneous distribution, since it is known that the observed improved function is generally limited to those cells that are located at the heterogeneous cell-cell interface (Bhatia *et al.* 1999). Fulfilling such requirement, the observed cell distribution of fibroblasts in rat co-culture (**Chapter III**) was homogeneous.

However, due to time limitations, in this work the co-culture strategies were not much explored in terms of cell characterization, interactions or even exploring other cell types. A good strategy to improve the cell culture model in terms of representativeness of the liver milieu can be to mimic capillary structures that could improve spheroids permeability to test compounds. This can be achieved by the addition of human umbilical vein endothelial cells (HUVECs) as has been done by Salerno *et al.* (Salerno *et al.* 2011) and Inamori *et al.* (Inamori et al. 2009).

In spite of the advances that the 3D approaches have brought to the Drug testing field, there are still some limitations that can prevent it from being a more predictable model of human risk. As an example, although the gel structure presents advantages for culturing cells it can also limit the prediction of drug induced cytotoxicity since the physico-chemical properties of the compounds can thwart them to reach the cells (Schutte *et al.* 2011). Moreover, 3D systems that combine encapsulation with hollow fibers for diffusion are also potentially limited by the fibers adsorbing hydrophobic drugs (Schutte *et al.* 2011) bringing an additional difficulty on the accurate identification of the drug concentration to which cells are subjected.

The use of cell spheroids by itself has as well some limitations; permeation of the compounds can be compromised being an important parameter to be controlled. In this work, the use of stirred systems was a way to facilitate the diffusion in spheroids, as well as the presence of other cells that can create channels; this was assessed biochemically on **Chapter IV** by the CDFDA method, and included on **Chapter V** by the *in silico* approach. This work also depicts the *in silico* approach where the results can be better integrated, extrapolated and understood.

The use of integrative (such as co-culture), multiparametric and multidisciplinary (such as *in vitro* + *in silico*) approaches can decrease the human risk in the clinical phases of drug development.

1.4. Stirring systems: why use it for the generation of hepatospheroids.

The generation of 3D cell constructs in fully controlled and monitored bioreactors creates a more homogeneous *in vivo*-like environment, and additionally decreases the biological cell-to-cell and culture-to-culture variability. Animal cells are cultured in bioreactors with the aim of maintaining well defined, physiological conditions while constituting a robust and reproducible approach (van Zijl and Mikulits 2010). The most relevant bioreactors used in this area are detailed in **Chapter I**; stirred tank vessels were selected for the work hereby presented.

The presence of stirring allows a better homogeneity of the medium and its flow can resemble the blood flow within the body while at the same time making it possible to overcome mass and gas transfer limitations. Comparing with other types of agitation, spinner stirring has shown to have a higher rate of aggregation (Wu *et al.* 1996). Additionally, with stirring it is possible to control the oversizing of spheroids avoiding the formation of necrotic cores (Moreira *et al.* 1995), that result from deprivation of nutrients and oxygen. This stirring can be performed with different types of impellers, as shown in **Chapter II**; being a versatile tool, it can be adapted to different cell types.

The spinner tank vessels are a very attractive tool for Pharma Industry as these are non-expensive, simple to work with, easy to transfer between laboratories and permit *in-vivo* like structures to be obtained. However, the work presented on this thesis has shown that the basal hepatocyte functionalities can be further improved when the cells are cultured in environmentally controlled bioreactors (**Chapters II** and **III**). With a tight and automatic monitoring and an on-line parameter control the cells have an homogeneous environment during culture time and a more robust model is obtained, which can be applied under GMP (Good Manufacturing Practices) conditions. Furthermore, the possibility to control several parameters, allows the performance of dynamic studies focusing on each one of them, which is not possible with the simple spinner tank vessels.

In the special case of hepatocytes, where the importance of oxygen control has already been highlighted, this bioreactor system not only allows such control but also presents the possibility of subjecting the cells to different oxygenation conditions to evaluate distinct responses (**Chapter III**). In this way, it is possible to mimic the perivenous and the periportal oxygen values as well as pathological liver perfusions (such as stroke or carcinoma). As a controversial issue, oxygen could have been further explored within the aim of this thesis, trying to study the rate perfusion that would maximize hepatic functionalities without inducing oxidative stress. Moreover, since cell cultures, especially primary cultures, show a dynamic metabolic profile, subjecting the cells to different oxygen levels during culture time could be a strategy to further extend culture time. However the optimization of these strategies would be too time-consuming within the scope of this work.

An additional advantage of these bioreactors regarding its application in Toxicology is the possibility to overcome the traditional bioreactor limitations to scale-up (Naughton 2002; Ratcliffe and Niklason 2002). Higher amounts of cells in the same conditions can be generated which, due to the non invasive and easy sampling, can be used to feed smaller models for high-throughput screening.

Furthermore, it is also possible to integrate a perfusion apparatus that will ensure the continuous renewal of nutrients and other factors as well as continuous removal of metabolic products with low culture perturbation, increasing the robustness of the system, as is described in **Chapter IV**.

Besides the advantages of the bioreactors used in this work, the big limitation is the minimum working volume. The system is proven to be compatible with high-throughput but it is not possible to run high-throughput studies in the bioreactor. The cells need to be harvest and being cultured for some hours/days in different surfaces/apparatus. Meanwhile, smaller vessels and prototypes are in development and hopefully will be commercialized soon.

Moreover, current strategies towards the accomplishment of *in vitro* ADME include the development of multi-organ systems – such as the IdMOC system (Xu *et al.* 2010) – simulating the whole living organism by having compartments with different cell types, hierarchically connected by culture medium. At the moment, this is just possible with miniaturized bioreactors (**subChapters 5.2.1** and **5.2.3** of **Chapter I**).

2. LOOKING AHEAD

The developed strategy can improve and extend the metabolic competency of hepatocytes for toxicological applications. We developed a cell culture model that can be used with reference toxicants in order to generate correlations between the data produced and the known *in vivo* values. Nevertheless the main advantage of these cultures, when further extended in time, is their application to chronic and repeated-dose toxicity assays.

2.1. Towards the accomplishment of the full replacement of animal models

In 2003, the 7th amendment to the European Union's Cosmetics Directive (76/768/EEC) has imposed a ban on the use of animals for testing Cosmetic compounds from 2009 onwards. In 2005, a team of experts has met and produced a report for DG SANCO (Directorate Generale for Health and Consumers) where it was stated that within the foreseen time it was not possible to generate alternative methods that could give accurate toxicity prediction (Coecke *et al.* 2005); thus the ban was postponed to 2013. Six years later, a similar exercise was performed (Adler *et al.* 2011), and in spite of still not having methods that can fully replace the use of animals, this accomplishment is expected in the near future. Since 2005 searching alternative methods to animal testing has highly developed, having generated several techniques. Nonetheless, integrative and multidisciplinary approaches are essential to reach full animal replacement.

The strategy developed in this thesis has generated a highly competent metabolic culture model that, when integrated with proper *in silico* strategies such as PBTK models, can predict human risk or/and microdosing. Nevertheless, this approach does not avoid the use of *in vivo* testing before approval, but will highly reduce the risk and animal testing Reduction and Refinement, within the 3R's policy.

The combination of different disciplines on the experimental design also allows the prediction of important mechanisms such as systemic pharmacological and toxicological paths. Multidisciplinary approaches and the generation of consortiums such as DNT (Neurodevelopmental Toxicology consortium) constitute growing initiatives (Vozzi *et al.* 2011) that aim at obtaining complete strategies that can accurately predict the human risk. It is important to have an active dialogue between engineers and biologists in order to further develop the adaptation of the cell environment to the cell sensitivities and needs.

3. CONCLUSION

With this work, by using the strategy of 3D spheroids in stirred tank vessels a robust model was obtained, that can generate liver-cultures closer to the *in vivo* and for periods of time further extended than those obtained with regular 2D cultures. Moreover, the system hereby developed presents technical characteristics that make it an attractive strategy for Toxicological applications. **Figure 6.2** summarizes the major accomplishments of the developed strategy.

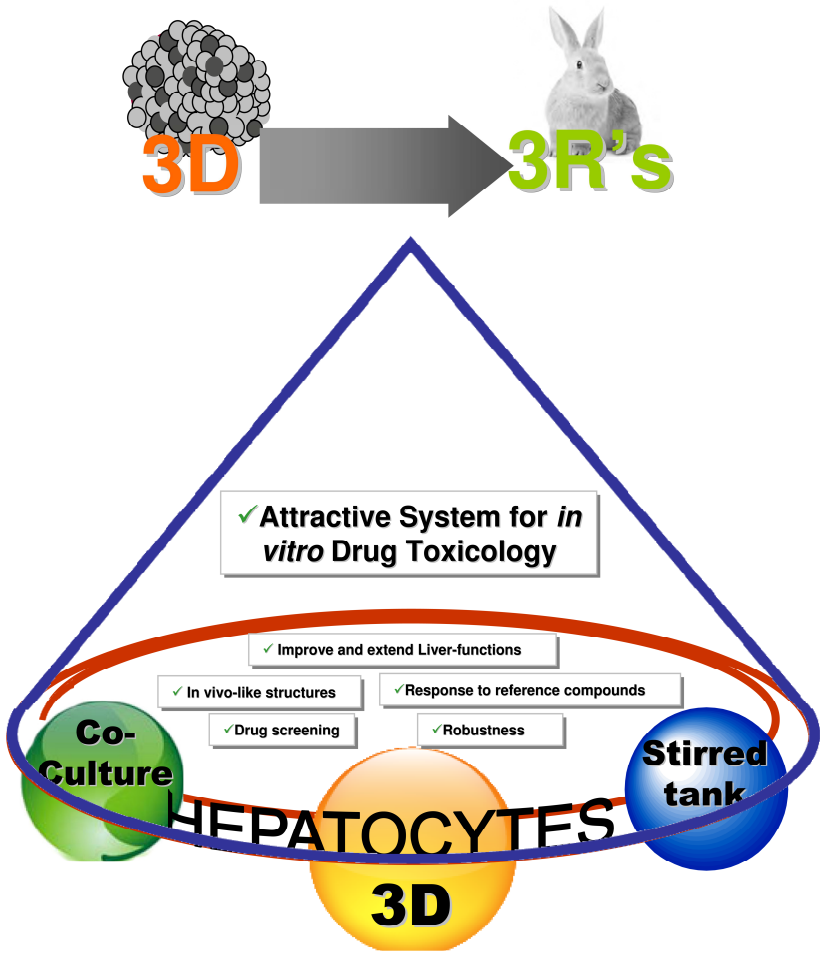

Figure 6.2: Schematic view of the focus and outcomes of the work developed in this thesis.

Each of the different approaches has disadvantages and limitations, but together they make important contributions to the generation of a robust and metabolically competent *in vitro* method for hepatocyte culture and towards its application on the Pharma Industry.

It is to be hoped that other research groups will further develop this system in order to achieve a complete 3R model.

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D. Hume (1711-1776)

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